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Newer analytical methods employed in examining blood, urine and cerebro-spinal fluid.

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NEWER ANALYTICAL METHODS EMPLOYED IN EXAMINING BLOOD, URINE AND CEREBRO-SPINAL FLUID

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MARTHA LOUISE NEUNER

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NAME OF STUDENT: Martha Louise Neuner

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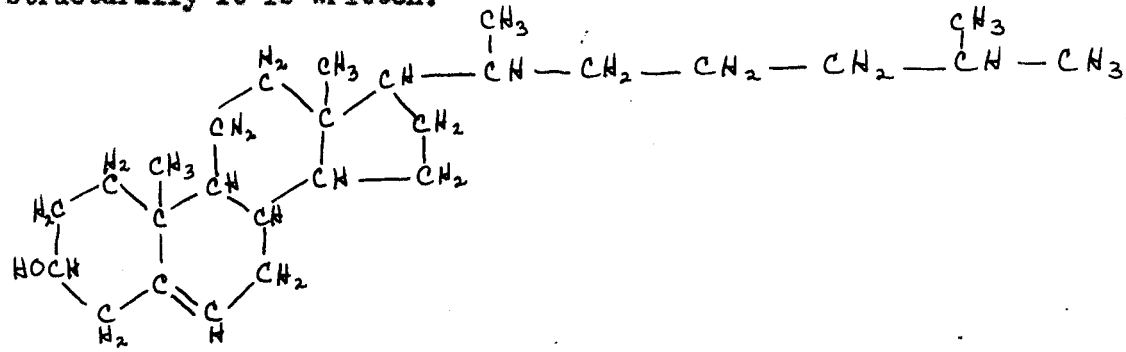
GENERAL INTRODUCTION

The object of this investigation has been threefold: first, to develop technique and facilitate speed and accuracy, second, to introduce some new and important clinical methods, third, to survey the development and inaccuracies of some important, newly-developing determinations of possible future clinical significance. The determinations included in this thesis are those that have been important in the past and are becoming increasingly more important to the University of Louisville Medical School Laboratory for the City Hospital, City Health Department and the Doctor's Research of Louisville.

A. DETERMINATION OF CHOLESTEROL IN BLOOD

INTRODUCTION AND HISTORY

Cholesterol is a simple lipide, a wax (cholesterol esters). Cholesterol is absorbed from the intestine, is a primary cell constituent and present in fairly large amounts in nervous tissue. It occurs free and combined as an ester. 99% in the brain tissue is in the free state. Cholesterol belongs to the class of sterols all of which have the cyclopentano-phenanthrene nucleus. It is a monatomic alcohol containing one double bond and has the formula $C_{27}H_{45}OH$. Structurally it is written:



It is soluble in ether, chloroform, benzene and hot alcohol. It crystallizes in thin, colorless plates. It is present in bile, biliary calculi, and blood. Cholesterol calculi and stones form easily. Cholesterol is held in solution in bile and by means of bile acids. It may be prepared from rabbit brain tissue by extracting with ether for one hour. (4).

The cholesterol present in the animal body has its origin from animal foods or through synthesis in the body from carbohydrate decomposition products. That cholesterol may be synthesized in the animal body is indicated by the fact that hens continue to lay eggs containing cholesterol when fed a cholesterol-free diet. Also the tissues of the rat contain the usual cholesterol content when fed a diet containing no sterols.

Cholesterol crystals have been but rarely detected in urinary sediments. When present they probably rise from a pathological condition of some portion of the urinary tract. Crystals of cholesterol have been found in the sediment in cystitis, pyelitis, chyluria and nephritis. Ordinarily they occur as large regular and irregular colorless, transparent plates, some of which possess notched corners. Frequently, instead of occurring in the sediment, cholesterol is found in a film on the surface.

Several qualitative tests for cholesterol are: (4)

1. Microscopic examination of crystals.

2. Sulfuric acid test (Salkowski) - Cholesterol, chloroform and concentrated sulfuric acid produce a bluish red to cherry red and purple in the chloroform layer and green in the acid layer.

3. Acetic anhydride - sulfuric acid test (Liebermann-Burchard) Cholesterol and chloroform plus ten drops of acetic anhydride and 3 drops of concentrated sulfuric acid give a color which varies from red through blue to bluish green.

4. Formaldehyde - sulfuric acid test. Chloroform, cholesterol and formaldehyde solution added to sulfuric acid solution (1 part of 40% to 50 parts of acid) gives a cherry red color in the chloroform. The chloroform is poured off and 2 drops of acetic anhydride added, a blue color results. This test is more delicate than the Salkowski test.

The determination of cholesterol is of great clinical importance in diagnosis. Cholesterol values normally range from 140-170 mg./100 cc. of whole blood, according to Bray (3). Normally it is about the same in plasma and whole blood. High values are obtained

in chronic nephrosis (335-830 mg./100 cc.), diabetes mellitus, lypemia, pregnancy, hypothyroidism, biliary obstruction (200-290 mg./100 cc.) and arteriosclerosis (210-230 mg./100 cc.). Low values (70 mg./100 cc.) result in pernicious anemia, hyperthyroidism, intestinal obstruction, infections and uremia. The changes are more marked with plasma or serum than with whole blood because the cells do not vary significantly in their cholesterol content.

THEORY AND METHODS

1. Determination of Total and Free Cholesterol in Blood Serum, Kaye's method. (5).

Reagents: 1. Total cholesterol standard. 100 mg. of dry cholesterol and 250 cc. of acetic anhydride (1cc. equals 0.4 mg. of cholesterol). Lasts for eight months. 2. Digitonin solution in glacial acetic acid. 175.6 mg. of digitonin in a little acetic are heated to 60°C., cooled, and diluted to 100 cc. with some solvent. Lasts 1 year. 3. Free cholesterol standard. 25 cc. of total cholesterol standard and 25 cc. of digitonin solution in glacial acetic acid and 25 cc. of acetic anhydride are mixed in a glass stoppered bottle, (3 cc. equals 0.4 mg. of cholesterol). Lasts 1 year. 4. Digitonin solution - 0.5%. 500 mg. of digitonin and 100 cc. of 50% alcohol (55 cc. of 95% alcohol and 45 cc. of distilled water) are heated to 60°C. Lasts 4 months. 5. Alcohol-ether solution. 750 cc. of 95% alcohol and 250 cc. of ether are mixed together. Keep in a cool place in a glass stoppered bottle. 6. Alcohol, 95%. 7. Concentrated sulfuric acid. 8. Ether. 9. Acetic anhydride. 10. Glacial acetic acid. 11. Chloroform. 12. Acetic anhydride-chloroform solution. Must be prepared immediately before use or stored in refrigerator for not more than two weeks. 10 cc. of acetic anhydride are mixed with 20 cc. of chloroform.

Method for total cholesterol: To 0.2 cc. serum in a 15 cc. centrifuge tube add 12 cc. of alcohol and ether solution in a rapid stream. Centrifuge three minutes at 2,000 revolution per minute. Decant into a 25 cc. Erlenmeyer flask. Evaporate just to dryness on a water bath. (Avoid overheating of dry residue. It is best to

evaporate the last trace of the solvent at 60°C.) When dry and cool add 3 cc. of acetic anhydride-chloroform solution (1:2) and 2 drops of concentrated sulfuric acid. Stopper, mix, and set aside in the dark for 10-15 minutes. Prepare the standard at the same time. To 1 cc. of cholesterol standard in acetic anhydride add 2 cc. of chloroform and 2 drops of concentrated sulfuric acid. Mix and let stand 10-15 minutes. Compare the two solutions in a colorimeter. Calculation of total cholesterol: Total cholesterol in mg./100 cc. of serum = $\frac{R_s}{R_u}$ x mg. cholesterol in standard x 500.

2. Cholesterol determination in blood, plasma, or serum; macro method of Homberger and Holtzheimer developed at the University of Louisville Medical School.

To 2 cc. of whole blood, plasma or serum in a 150 cc. Erlenmeyer flask add 20 cc. of 25% NaOH. Reflux on a water bath for two hours and cool. Extract 6 times by shaking with 50 cc. of ether for five minutes, washing each ether extraction in a separatory funnel with not more than 5 cc. of water. Combine all ether extracts in a dry 500 cc. Florence flask. Distill off ether from combined ether extract. Use boiling water to heat for distillation. Heat the residue remaining after the ether distillation to dryness in an oven at a temperature of 60-100°C. (not above 100°C). Cool. Take up the dried residue in 10 cc. of chloroform. Add the chloroform directly to the Florence flask containing cholesterol residue, shaking vigorously while stoppered. Pipette out a 5 cc. portion of chloroform extract and place in a dry 150 cc. Erlenmeyer flask. Stopper with rubber stopper. To another dry 150 cc. Erlenmeyer flask add 5 cc.

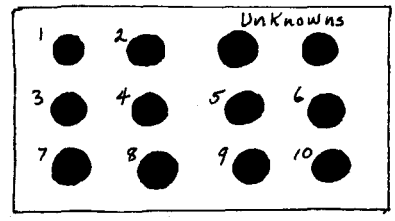
cholesterol standard (1 cc. = 5.0 mg. of cholesterol). To standard and unknown each add 2 cc. of acetic anhydride then 0.2 cc. of concentrated sulfuric acid. Place immediately in a cold dark place for 15 minutes. Compare in the colorimeter.

3. Approximate value of serum or plasma cholerestol by Wabnitz micro method.

Procedure: To 2 cc. of alcohol-ether solution in a 15 cc. Pyrex centrifuge tube add 1 drop of blood (serum, plasma, or whole blood). Place in a water bath at 60°C. for 1 minute and then centrifuge for 1 minute. Decant into spot plate and allow to evaporate at 60°C. in an oven. To residue left in tube add 1 cc. alcohol-ether solution and centrifuge for 1 minute. Decant into same spot and evaporate at 60°C. (not over 60°C.). To the dry residue add 10 drops of chloroform and 10 drops of acetic anhydride and mix by agitation. Prepare the standards and add 2 drops of concentrated sulfuric acid to each standard and to unknowns. Cover with glass. Let stand for 10 minutes and compare. Read the value from the chart. Normal serum-plasma contains approximately 0.075-0.125 mg./0.05 cc. (1 drop). Standard cholesterol solution (in acetic anhydride) contains 0.02 mg./0.05 cc. (1 drop).

#	cholesterol std. sol. drops	acetic anhydride drops	$CHCl_3$ drops	actual cholesterol content in mg.	represents mg./100 cc.
1	1	9	10	0.02	40 Low value
2	2	8	10	0.04	80 Low value
3	3	7	10	0.06	120 Normal value
4	4	6	10	0.08	160 Normal value
5	5	5	10	0.10	200 Normal value
6	6	4	10	0.12	240 Normal value
7	7	3	10	0.14	280 High value
8	8	2	10	0.16	320 High value
9	9	1	10	0.18	360 High value
10	10	0	10	0.20	400 High value

Arrange standards as follows on white porcelain spot plate.



Add cholesterol standard to each as in the chart. Add acetic anhydride and chloroform (cover with glass). Add the sulfuric acid (2 drops to each spot) and mix by rotary motion of the spot plate.

Reagents: 1. Concentrated sulfuric acid. 2. Chloroform. 3. Acetic anhydride. 4. Standard cholesterol. 0.04 gm. of cholesterol in 100 cc. of acetic anhydride. 5. Alcohol-ether solution. 3 volumes of 95% alcohol to 1 volume of ether.

4. Cholesterol Determination in Blood or Serum, micro method of Wabnitz developed at the University of Louisville Medical School.

To 0.2 cc. of blood or serum in 15 cc. centrifuge tube add 12 cc. alcohol-ether solution in a rapid stream. Centrifuge 3 minutes at 2,000 revolutions per minute. Decant into a 25 cc. Erlenmeyer flask. Evaporate just to dryness on a water bath. (Avoid over-dryness. It is best to evaporate the last trace of the solvent at 60°C. in a sand bath.) Thoroughly dry and cool. Add 3 cc. acetic anhydride-chloroform solution (1:2) and add 2 drops of concentrated sulfuric acid. Stopper, mix and set aside in dark 10-15 minutes. Compare in a colorimeter with 1 cc. cholesterol standard in acetic anhydride plus 2 cc. of chloroform and 2 drops of sulfuric acid.

Reagents: Alcoholic-ether solution. To 250 cc. of ether add 750 cc. of 95% ethyl alcohol. Standard for total cholesterol. Dissolve 100 mg. of C.P. dry cholesterol in 250 cc. acetic anhydride (1 cc. = 0.4 mg. cholesterol). Lasts at least 8 months.

Calculation: Total cholesterol mg./100 cc. = $\frac{R_s}{R_u}$ x mg. of cholesterol in standard x 500.

5. Cholesterol Determination Blood, Guy-Sackett method.

In a 15 cc. graduated centrifuge tube add 9 cc. 95% alcohol, 3 cc. of ether, mix by inversion, to 0.2 cc. of blood, run in slowly. Cork tightly, shake vigorously for about 1 minute and let lie horizontally with sediment evenly distributed along the tube for 30 minutes. Centrifuge rapidly for 3 minutes to remove the precipitated protein. Decant the fluid into a small beaker. Evaporate just to dryness in a water bath. (Be very careful to avoid the danger of fire.) Extract the cholesterol twice for about 2 minutes each time with small portions (2.0-2.5 cc.) of chloroform and decant into a graduated centrifuge tube. Cool, if necessary; make up to 5 cc. with chloroform. Transfer 5 cc. of standard cholesterol in chloroform (containing 0.4 mg. cholesterol) to a similar tube. Add to standard and unknown 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid. Mix by repeated inversions. Set aside in the dark for 15 minutes. Compare at once.

Calculation: (Standard set at 15) $\frac{15}{R}$ x 0.4 x $\frac{100}{0.2}$ mg. in 100 cc. of blood.

6. Blood cholesterol - Lieboff Method (3).

Place a fat free filter paper disc on the shoulder of the Lieboff extraction tube graduated at 5 cc. Place 0.25 cc. of oxalated blood on the disc and incubate one half hour to dryness. Run 5 cc. chloroform on the blood, attach tube to a reflux condenser, and place in a water bath kept at 75°C. Be sure that the water level is above

that of the chloroform and that the condensed chloroform drips upon the blood. Extract for one half hour. Cool tube in water and detach, remove disc and add chloroform to the 5 cc. mark. To a similar tube add 5 cc. standards, containing 0.4 mg. of cholesterol. To each tube add 2 cc. purified dry acetic anhydride and 0.1 cc. concentrated sulfuric acid. Cork, mix, cool in ice box for 10 minutes and compare in colorimeter. Set standards at 10 or 15 mm.

Calculation: $\frac{S}{R} \times 160 =$ mg./100 cc. Normal values range from 140 to 170 mg./100 cc. whole blood.

7. Determination of Cholesterol, Reinhold and Shiels' (7), modification of the Myers-Wardell Method.

Procedure: Pipette 1 cc. of blood plasma or serum into a small mortar containing about 8 gm. of anhydrous sodium sulfate. Mix uniformly, dry at 100°C. for 10 minutes, cool in a dessiccator, pulverize and transfer completely to a paper extraction shell which is inserted into a Soxhlet extraction tube, suspended from the condensing coil of the extraction apparatus. Place 20-25 cc. of redistilled anhydrous chloroform in the extraction flask. Continue extraction on an electric hot plate for 90 minutes, during which time cold water flows through the condenser or coil. Cool the chloroform extract, transfer to a 25 cc. volumetric flask and make up to volume with rinsings of fresh chloroform. Then filter the well-mixed extract if necessary. Carry out the colorimetric estimation as follows: Pipette 10 cc. of the chloroform extract into a dry test tube, and add 2 cc. of a freshly prepared, warm, 10:1 mixture of acetic anhydride and concentrated sulfuric acid. Treat 10 cc. of a standard solution of

cholesterol in chloroform in exactly the same way. After thorough mixing, keep the solution at 25°C. for exactly 30 minutes, in the same light by which the colorimetric readings are to be made. If the original Duboscq colorimeter is used, it is necessary that the cups be remounted in plaster of Paris instead of balsam.

Calculation: $\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 0.8 \times \frac{100}{0.4}$ = mg. cholesterol/100 cc. blood.

8. Determination of Cholesterol - Method of Myers and Wardell (1,2,6).

Procedure: Place a small piece of fat-free cotton in the extractor, tamping it with a glass rod. The upper surface of the cotton should be about 2.5 cm. from the open end of the extractor. Draw up 0.2 cc. of blood, plasma or serum into a pipette. Place the tip of the pipette against the upper surface of the cotton and press it to a fairly firm seat. The sample will be absorbed by the cotton quantitatively. Place another small plug of cotton in the extractor and press it down firmly against the first piece that has soaked up the sample. Insert a piece of heavy walled rubber tubing into the open end of the extractor and connect the other end of the tubing to a filter pump. Place the extractor with the tubing attached in a dry test tube immersed in boiling water. Draw a current of air through the warmed sample for 15 minutes to dry it. Fill the bulb of the extraction tube with dry chloroform and place the extractor in it. Support the extraction tube over the micro hot plate or other source of moderate heat and place a mushroom condenser in its mouth. Turn on the heat and regulate the current so that the chloroform is heated

very gently. Otherwise, it may spurt out of the tube since it is easily superheated beyond its boiling point. If some chloroform does bump out of the tube at the start, the determination is not spoiled but the lost solvent must be replaced. After boiling has begun, increase the current so that the extractor is kept full of the condensate that falls from the tip of the condenser. Permit the extraction to continue for an hour. The apparatus will require no attention during this period. Turn off the current and remove the condenser and extractor from the extraction tube. Drop the extractor into a beaker of warm water which soaks into the cotton and facilitates its subsequent removal. Stopper the extraction tube and cool it under the tap. Add chloroform to the 5 cc. graduation. Place 5 cc. of cholesterol standard solution in a 10 cc. glass stoppered graduated cylinder or test tube. Add acetic anhydride to both tubes to the 7 cc. mark. Add 0.1 cc. of concentrated sulphuric acid to each tube. Stopper the tubes firmly and shake them vigorously for a few moments. Place the tubes in the refrigerator set at 10°C. Compare immediately. Total time for the determination, 1 to 2 hours.

Reagent: Cholesterol standard solution. Dissolve 0.080 gm. pure cholesterol in dry chloroform and dilute to a liter with the same solution. Preserve in an absolutely tight bottle.

Calculation: Mgs. cholesterol per 100 cc. of blood (or plasma or serum) = $\frac{S}{U} \times 200$.

Rose, Schattner, and Exton (8) describe a method for cholesterol using Tschugaeff rose red color. They claim it is just as accurate as the Liebermann-Burchard method and the rose red color is easier to compare in a colorimeter.

CONCLUSIONS

Since the determination of cholesterol is of great clinical importance in diagnosis and sice more cholesterol determinations than nay other single determination come into the University of Louisville Medical School Laboratory there is need for a rapid accurate method.

The macro-method developed by Homberger and Holtzheimer at this laboratory gives reproductible results but requires six hours to complete one determination. The micro-method developed by Wabnitz using a colorimeter is less accurate but requires only one and one-half hours to perform. The micro-method developed by Wabnitz for determining approximate values (using a spot plate) gives values between those obtained with the above macro and micro methods. His approximate method cuts the time down to less than one hour. It can be used where only approximate values are required and thereby saves considerable time.

The method of Myers and Wardell is also a micro-method and gives considerable trouble in the process of extracting the cholesterol from the whole blood.

For the purposes of the University of Louisville Medical School Laboratory the macro-method of Homberger and Holtzheimer or the micro-colorimetric method of Wabnitz are the most accurate. For speedy determinations or checking, Wabnitz spot plate method can be used.

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B. DETERMINATION OF VITAMIN C (ASCORBIC ACID) IN BLOOD AND URINE

INTRODUCTION AND HISTORY

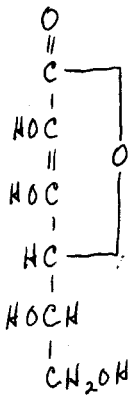
Vitamin C is concerned specifically with the maintenance of intercellular substances, including the collagen of fibrous tissue structures, the matrices of bone, cartilage and dentin, and all non-epithelial cement substances. In the absence of the protection afforded by this vitamin the condition known as scurvy develops.

The onset of this disease is gradual in humans, a period of indolence, fleeting pains in the joints, and shortness of breath being followed by decline in weight and anemia. Soon the complexion becomes sallow, subcutaneous hemorrhages occur upon slight injury, the gums become spongy and bleed easily, the teeth become loose and fragile, bones fracture readily, and often there is marked edema of the extremities. Hemorrhage is the predominating feature of the disease, and when it occurs internally, it is frequently the cause of death. A response to treatment for scurvy is noted frequently within a few days and complete cure within two weeks is not uncommon.

The earliest signs of scurvy are changes in the teeth and gums and are the basis of several methods of determining Vitamin C deficiency. However, the ascorbic acid content of the blood or urine is much more dependable criteria for measuring the state of vitamin C nutrition. The normal level of ascorbic acid in the blood is 0.8-2.4 mg./100 cc. of whole blood (average 1.6 mg.). In cerebro-spinal fluid the amount is usually 1.75-2.0 mg./100 cc. The renal threshold is about 1.4 mg./100 cc.; the daily may range from 15 to 25 mg., depending upon the adequacy of the diet. The human daily requirement for ascorbic acid may be regarded as approximately 5 mg. (100 International Units) for infants, 15 mg. for adults and 25 mg. for pregnant

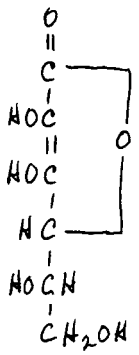
or lactating women.

Crystalline vitamin C was first isolated from lemon juice in 1932 by King and Waugh. (17). l-ascorbic acid, $C_6H_8O_6$, has the following structural formula:



THEORY AND METHODS

l-Ascorbic acid, $C_6H_8O_6$, has the following structural formula:



It is a white, crystalline powder which turns slightly yellowish on standing. It is optically active, soluble in water and alcohol, insoluble in benzene and ether, and melts at approximately 192°C. It is easily oxidized in solution on exposure to air, instability increasing with alkalinity and in the presence of light. Ascorbic acid is a powerful reducing agent; its capacity to reduce silver nitrate (to metallic silver), iodine, ferricyanide, methylene blue, and 2-6-dichlorophenolindophenol, serving as the bases of methods for its chemical estimation. (14).

The average fecal content on unsupplemented diet is 5 mg. daily. The procedure and calculation for determining vitamin C in feces is given by Chinn and Farmer (6) using the 2-6-dichlorophenol-indophenol method.

There is a definite relation between the level of reduced ascorbic acid in the blood and the amount excreted by the kidneys; when the former exceeds 1.4 mg./100 cc., urinary excretion increases. This fact is utilized in the excretion test of Goldsmith and Ellinger (12), a method of determining the state of vitamin C nutrition which

is applicable to ambulatory patients and requires only a few hours to perorm. Indophenol tablets equivalent to 1 mg. of ascorbic acid were used and the 6 hour excretion test was found to be as good as the 24 hour urinary excretion.

A method for determining Vitamin C in urine has been developed by Roe and Hall (23). Ascorbic acid is oxidized with norit and the dehydroascorbic acid is separated as a 2-4-dinitrophenylhydrazine derivative. This derivative is decomposed by boiling with HCl containing $SnCl_2$ with the formation of furfural. The furfural is determined colorimetrically by the aniline-acetate method. The method is called the osazone-furfural method.

A method using the photoelectric colorimeter has been described by Evelyn, Malloy, and Rosen (18). In the method described the titration has been replaced by an objective photoelectric measurement of the amount of dye decolorized when a measured quantity of urine reacts with an excess of dye. This method does not require standardization of the dye solution, eliminates errors due to interfering colored substances, and allows the measurement to be completed within 5 seconds after addition of the dye. This greatly reduces errors due to non-ascorbic acid reducing substances, and a simple extrapolation procedure makes it possible to reduce the error still further.

In the determination of ascorbic acid in tissues and urine, Roe (24) claims his fuffural method is more specific than the indophenol method. The method consists essentially of the determination of the furfural formed by boiling an extract of a tissue in which the ascorbic acid has been oxidized by passage through norit, with

HCl alone and with HCl containing $SnCl_2$. The value obtained with the HCl-$SnCl_2$ mixture minus that given by the HCl alone is the amount of furfural from ascorbic acid. Furfural is determined by the color formed with aniline, stabilized with $SnCl_2$ and proper amounts of acetic acid.

A skin test described by Rotter (German) was studied and found to be unsatisfactory for the determination of the state of vitamin C nutrition (13). The test is based on the time of decolorization of an intradermal injection of 2-6-dichlorophenolindophenol.

Kassan and Roe (16) have discussed the preservation of ascorbic acid in drawn samples of blood. If blood plasma is separated from the red cells and allowed to stand, its ascorbic acid content decreases. To obtain correct plasma ascorbic values and to preserve the ascorbic acid in blood for a time, it is necessary to prevent hemolysis completely. Intact red cells have a preservative effect on the ascorbic acid of the plasma in which they are suspended. Maintenance of conditions which preserve the intactness of the red cells has been found the most effective procedure for stabilizing the ascorbic acid content of drawn samples of blood. Pyrex, paraffin, and collodion-lined tubes used as blood containers gave a better preservation of the ascorbic acid of plasma than ordinary glass containers. There was found to be no advantage in using KCN as a preservative as suggested by Pijoan and Klemperer.

Canned fruit juices were examined by the Council on Foods (7) and found to have the following vitamin C content per 100 cc.

Pineapple juice	300 International Units

Tomato juice	300 International Units
Grapefruit juice	400 International Units
Orange juice	900 International Units
Lemon juice	1000 International Units

Canned orange juice is only slightly lower in vitamin C than fresh orange juice. Good intake for adults is 1000 I.U. per day, and good intake for children is 2000-3000 I.U. per day. 100-200 I.U. will prevent scurvy in children and 300 I.U. will prevent the disease in adults.

Vitamin C Beverage solution was found by the Council on Foods (8) to prevent loss of vitamin C of orange juice on standing in ice box for 24 hours. However, the orange juice stabilized the V-C-B solution more than it did the orange juice. The V-C-B solution was prepared with concentration of 10.49 gm. dry base/100 cc. of solution, which is equivalent to 3½ oz. of the powder per quart. Equal parts of the orange juice and V-C-B were used. The V-C-B powder is manufactured by Hilker and Pletsch Company, Cincinnati and Chicago.

The relation of reducing value and extent of browning to the vitamin C content of orange juice exposed to air was studied by Joslyn, Marsh, and Morgan (15). The decrease in iodine value was followed by titration of 50 cc. of juice with 0.01 N iodine solution, starch being used as an indicator. The end point chosen was that when the starch iodine color persisted for 15-30 seconds.

The most widely studied method adapted to the determination of ascorbic acid is that of Tillmans based on the reduction of 2-6-dichlorophenolindophenol (or 2-6-dichlorobenzenoneindophenol) in

acid solution. The chief disadvantage of this and other methods lies in the fact that they are not specific, and precautions must be observed lest substances like glutathione, phenolic compounds, cysteine, H_2S, and products of alkaline decomposition of sugars, interfere.

Tauber, Kleiner and Mishkind, (27) describe a method using a specific, ascorbic acid oxidase. For purposes of approximation, 0.01 N iodine solution may be employed (unless other reducing substances are present). One cc. 0.01 N iodine = 0.88 mg. ascorbic acid.

METHODS

1. Purification of the Indophenol dye. (14).

To 5 gm. Na 2-6-dichlorobenzenoneindophenol in a beaker cooled in an ice bath, add 2.25 gm. phenol and stir thoroughly. Add 18 cc. 3 N NaOH slowly and with constant stirring, keeping the temperature near 0°C. After one-half hour add 150 cc. 15% NaCl, stir and filter with suction. Redissolve the crude dye in 7-10 successive 300 cc. portions of warm, distilled water, and again salt out while stirring. Filter and dry in a vacuum dessiccator. Standardized tablets of the dye (1 tablet equivalent to 1 mg. ascorbic acid) are prepared by Hoffman-La Roche, Nutley, N.J.

2. Preparation of the Indicator solution. (14).

Place 0.1 gm. of the purified dye in a prepared filter and dissolve with successive portions of warm water, collecting the filtrate in a 200 cc. volumetric flask. Cool and make up to the volume of the flask. Keep on ice and discard after 2-3 days. Standardize daily against pure ascorbic acid. (one cc. is equivalent to approximately

0.12 mg. ascorbic acid.)

3. Preparation of standard ascorbic acid solution. (14).

Weigh out 60 mg. of ascorbic acid and dissolve and dilute to 100 cc. with 5% acetic acid. Dilute 2 cc. of this solution to 50 with 5% acetic acid. Now determine the ascorbic acid equivalence of the dye solution by titrating against 2 cc. portions of the ascorbic acid solution. The ascorbic acid may be further checked by titrating against 0.01 N iodine solution.

4. Vitamin C determination in Orange juice, Indophenol method of Wabnitz. (26).

Reagents: 1. Indophenol dye (0.0005 gm./cc.). Keeps 2 to 3 days. Dissolve 0.0125 gm. dye in 25 cc. hot water and refrigerate. 2. Ascorbic acid stock solution (1 mg./cc.). Prepare daily. Dissolve 0.025 gm. in 25 cc. water. 3. Ascorbic acid standard solution (0.1 mg./cc.). Prepare daily . Dilute 1 cc. of stock solution to 10 cc. 4. Standardization of Indophenol dye. Titrate 1 cc. or a 1 cc. standard ascorbic acid solution with Indophenol dye in acid solution (i.e., e.g. 1 cc. standard ascorbic acid plus 5 cc. H_2O plus 1 drop dilute HCl.) Titrate immediately with dye to first permanent pink end point.

Method for orange juice: To 1 cc. (or 0.1 cc.) orange juice plus 5 cc. H_2O plus 1 drop dilute HCl. Titrate with standard dye solution. 1 International Unit Vitamin C = 0.05 mg. ascorbic acid = 0.1 cc. lemon juice.

5. Determination of Vitamin C, method of Bessey and King (5).

To prepare indicator solution dissolve 0.10 gm. of dry dye

with successive portions of warm water, cool, add water to make up the volume to 200 cc. and filter. Add a small amount of phosphate buffer (pH 6.8) and keep in dark bottle. To 5 cc. of fresh strained lemon juice add 0.01 N iodine solution containing 15 gm. KI per liter until a permanent bluing of the starch indicator results. Each cc. of 0.01 N iodine represents 0.88 mg. vitamin C (lactone form). A separate 5 cc. portion of the juice is titrated with the dye solution to a permanent pink. The iodine titration permits calculating the vitamin C content of the lemon juice end from this the value of the dye solution can be calculated in terms of mg. of vitamin C. The dye and vitamin C react mol for mol:

$$\underset{\text{(Vit.C)}}{C_6H_8O_6} + \underset{\text{(blue in alkaline red in acid)}}{O{:}C_6H_2Cl_2{:}N.C_6H_4ONa} = C_6H_6O_6 + \underset{\text{(colorless)}}{HO.C_6H_2Cl_2.NHC_6H_4ONa}$$

6. Ascorbic acid determination in deproteinized blood plasma. (11).

Method: Place a 2 cc. oxalated plasma in a centrifuge tube. Add 4 cc. distilled water and 4 cc. 5% meta-phosphoric acid. Mix thoroughly and centrifuge. Transfer 2 cc. portions of the fluid into test tubes and titrate with the standardized solution of 2-6-dichlorobenzenoneindophenol using a microburette. The first permanent pink color is the end point.

Calculation: Titration x ascorbic acid equivalents (in mg.) per cc. dye solution x 100/0.4 = mg. ascorbic acid per 100 cc. plasma.

Interpretation: The level of reduced ascorbic acid in normal human blood depends on dietary intake. Values below 0.75 mg. indicate subnormal ascorbic intake; values below 0.50 indicate

clinical scurvy.

7. Determination of ascorbic acid, modified method of Tillmans and Hirsch. (1).

Method: Dissolve 1 standardized tablet of the dye, 2-6-dichlorophenolindophenol, in water and dilute to 50 cc. It is completely soluble if 2 drops of ammonium hydroxide are added. Pipette 5 cc. plasma into a 15 cc. centrifuge tube, add 10 cc. 5% trichloracetic acid, stopper, shake, and centrifuge. Decant all of clear solution into a graduated tube, and note volume of solution (V). Pipette 10 cc. of trichloracetic acid into a second tube, dilute to 15 cc. with water. Titrate both tubes with the dye solution. Run the reagent into the solution until a pink color persists for 30 seconds.

Calculations: $\frac{6}{V}$ = x U (cc. for unknown) - 0.4 B (cc. for blank) = mg. ascorbic acid/100 cc. blood.

Interpretation: The ascorbic acid in plasma is not decreased much except in marked scurvy. For subclinical scurvy, it is better to use urine or to perform a tolerance test. The blank corrects for reducing substances in the trichloracetic acid.

8. Determination of blood ascorbic acid, method of Pijoan and Klemperer. (22).

Method: Ascorbic acid is determined clinically in serum. Add 5 mg. KCN and 10 mg. of potassium oxalate to centrifuge tubes along with 6 cc. blood and centrifuge. Transfer 2 cc. of plasma to a centrifuge tube and add 2 cc. distilled water and 6 cc. of 10% metaphosphoric acid (made up daily). Stir rapidly, allow to

stand for three minutes and centrifuge. Place two cc. of the supernatant fluid in a beaker and titrate to a pink end point with 2-6-dichlorophenolindophenol, using a light with a dayligh filter. In preparing the dye solution it is convenient to dissolve it in water heated to 85°C, shake 15 minutes, filter if necessary, and standardize against pure ascorbic acid.

Calculation: cc. dye used in titration $\times \frac{0.001 \times 176 \times 100}{0.4}$ = cc. dye used x 44 = mg. ascorbic acid/100 cc. plasma.

0.001 is concentration of dye in millimols/cc.

176 is mg. ascorbic acid equivalent to 1 millimol

0.4 is amount of serum used for titration

The whole is multiplied by 100 to give the results in terms of 100 cc. of plasma. The error in the titration amounts to $\pm$ 0.1 mg.

9. Ascorbic acid content of blood, method of Farmer and Abt. (10).

Method: Draw 5 cc. blood and oxalate it, centrifuge, and prepare tungstic acid filtrate from plasma. Pipette 2 cc. plasma into centrifuge tube and add 4 cc. distilled water and 2 cc. 5% Na tungstate. Mix, then add 2 cc. 1/3 N H_2SO_4, mix, allow to stand 2 minutes and centrifuge. Pipette 2 cc. portions into test tubes and titrate immediately to the first pink end point (compared with untitrated solution). A 5 cc. microburette is used to measure the required volume of dye solution.

Reagents: Indophenol dye. Extract approximately 0.1 gm. of sodium 2-6-dichlorobenzenoneindophenol twice with boiling water and pour through small filter paper. Dilute to a volume of

50 cc. This solution is good for three weeks as stock solution. Dilute 10 cc. stock solution to 100 cc. with distilled water. Standardization. Weigh out 60 mg. ascorbic acid, dissolve in 5% acetic acid solution. Now titrate the dilute dyestuff against 2 cc. portions of dilute ascorbic acid solution in the manner prescribed for titration of deproteinized plasma solution. Check the ascorbic acid solution further by titration against a 0.01 N iodine solution (1.14 cc. of 0.01 N iodine = 1 mg. ascorbic acid) which has previously been standardized against potassium iodate. The ascorbic acid solution must be used immediately after standardization.

10. Estimation of ascorbic acid in blood serum and plasma, method of Taylor, Chase, and Faulkner. (28).

Method: Make two samples. Place 2 cc. blood serum or plasma into 50 cc. centrifuge tubes, add 14 cc. H_2O, 2 cc. 5% Na tungstate, 2 cc. 1/3 N H_2SO_4, mix, allow to stand, and centrifuge. Decant into flasks, add 0.5 cc. glacialacetic acid, stopper, and keep in the dark. To these flasks add subsequent washings. To the protein precipitate add 2 cc. 5% Na tungstate, stir and dilute with 14 cc. water, reprecipitate as before, centrifuge and add liquid to flasks. Repeat 4 times. Titrate the contents of the flasks with Tillman's reagent (prepared and standardized as below), 1 cc. = 0.10 cc. ascorbic acid. Carry out rapidly to the standard end point, the color produced by 1 drop Tillman's reagent added to boiled distilled water of same volume as unknown and containing 0.5 cc. glacial acetic acid. The end point to match standard is

that which will persist for 1 minute or more. The blank (not more than 0.005 mg. ascorbic acid) is deducted from value obtained.

Reagents: 1.5% Na tungstate prepared fresh weekly. 2. 1/3 N H_2SO_4 prepared fresh weekly. 3. Glacial acetic acid. 4. Ascorbic acid containing 1 mg./cc. 5. 2-6-dichlorophenolindophenol containing 0.2 mg./cc. 6. Starch indicator. 7. 1/100 N solution of iodine in KI. 8. Standard ascorbic acid solution. Dissolve 100 mg. ascorbic acid in 100 cc. boiled distilled water. Check daily by 1/100 N iodine solution. Keep in brown bottle in ice-box. 9. Tillman's reagent. Dissolve 20 mg. 2-6-dichlorophenol-indophenol in 75 cc. boiled distilled water, cool and dilute to the mark. Keep in icebox and standardize daily against ascorbic acid solution; it lasts two weeks.

11. Ascorbic acid in urine, method of Harris and Ray. (14).

Method: The urine may be titrated immediately or preserved with 10% of its volume of glacial acetic acid. Pipette 0.05 cc. 2-6-dichlorophenolbenzenoneindophenol (standardized by titration with a solution of pure ascorbic acid so that 0.05 cc. is roughly equivalent to 0.025 mg. of ascorbic acid.) To a centrifuge tube, add 2 drops of glacial acetic acid and titrate with the urine from a micro-burette, within 2 minutes. If more than 2 cc. of undiluted urine are required, the urine may be considered free of ascorbic acid.

Interpretation: Normal individuals show a marked rise in vitamin excretion following oral doses, while individuals with vitamin C deficiency or scurvy show little change. Adults on

average diets may excrete about 25 mg./day. Ascorbic acid may be administered in the form of orange juice which may be analyzed as directed above. Fresh orange juice contains about 0.5-0.6 mg. ascorbic acid per cc.

12. Determination of ascorbic acid in urine, method of Tillmans. (1).

Reagents: To prepare the dye solution, dissolve 1 tablet 2-6-dichlorophenolindophenol in water and make up to 50 cc. One cc. of the solution is equivalent to 0.02 mg. of ascorbic acid and the solution is sufficiently accurate without standardization.

Method: Filter the urine and pipette 20 cc. into flask containing 2 cc. glacial acetic acid. Add the dye solution from a micro-burette rather quickly until a pink color lasting for 30 seconds is obtained.

Calculations: Mgs. ascorbic acid per liter of urine = cc. of dye solution used to titrate 20 cc.

13. Determination of vitamin C with methylene blue, method of Matini and Bonsignore. (19).

Method: Triturate approximately 0.5 gram of tissue with washed sand in a mortar, extract with 8% trichloracetic acid solution, centrifuge and filter. Wash the residue, the mortar, and the filter twice with the trichloracetic acid and make the filtrate to 25 cc. Add to 5 cc. of the extract 2 cc. of a solution of 30 grams sodium citrate, and 8 grams sodium bicarbonate in 200 cc. water and 1 cc. 5% sodium thiosulfate solution. In a second tube place 8 cc. of water. To each tube then add 0.2 cc. of a

1:10,000 solution of methylene blue and expose the tubes to intense sunlight. In the presence of ascorbic acid the solutions are decolorized. Add more methylene blue to the unknown until after exposure its color matches the control.

Calculation: The number of cc. of methylene blue used multiplied by the amount of dye in 1cc. and divided by the weight of the sample extracted gives the amount of ascorbic acid per gram of tissue. One cc. of methylene blue (1:1,000) is equivalent to 0.047 milligrams ascorbic acid on the assumption that ascorbic acid reacts with methylene blue mol per mol.

The exact titer of methylen blue in terms of ascorbic acid can be determined by using a pure solution of ascorbic acid or of lemon juice. To 1 cc. of clear lemon juice, diluted to 15 cc. with water, are added 0.005 N I_2 until a faint blue color is visible on the addition of starch. A second sample of the lemon juice (1 cc.) is diluted to 50 cc. with 8% trichloracetic acid and 5 cc. of this solution treated with methylene blue as previously described. The number of milligrams of ascorbic acid corresponding to 1 cc. of methylen blue is obtained from the formula: $X = \frac{.44\ A}{10\ B}$ where A is the number of cc. I_2 used and B the number of cc. of methylene blue used. The leuco base of methylene blue is not oxidized spontaneously upon exposure to air due to sodium thiosulfate and sodium bicarbonate present.

Interpretation: Concordant and satisfactory results have been obtained by different workers. It is claimed that the method is superior to the dichlorophenolindophenol procedure, because of the sharper end-point, the rapidity, and the fact that it is

not affected by the presence of other reducing substances, (glutathione and cysteine).

CONCLUSIONS

In reviewing the literature it was found that the ascorbic acid in plasma was not decreased much except in marked scurvy. For subclinical scurvy it is better to determine the ascorbic acid in the urine or to perform a tolerance test. The analysis of blood has the further disadvantage of requiring a large sample. The methods should be applied to plasma which has been separated from the cells as promptly after withdrawal as possible.

The three most frequently used methods are the iodine method, the methylene blue method and the Na-2-6-dichlorobenzene-oneindophenol dye method. The iodine method is used by the makers of orange beverages in determining vitamin C because it gives slightly higher results than the indophenol dye method. The indophenol dye method, modified by Wabnitz at the University of Louisville Medical School laboratory, is quite accurate, results checking almost perfectly. However when determinations are needed in a short time the method is inadequate because the dye has to be prepared and standardized with pure ascorbic acid fresh each day. If ample time is available the method is simple and more accurate than the iodine method. The iodine method is affected by the presence of other reducing substances such as glutathione and cysteine, while the indophenol dye method and the methylene blue method are not affected to such a great extent. The methylene blue method is claimed to be superior to the indophenol dye method because its end point is sharper and because of the rapidity of the procedure, and because of the fact that it is not affected by the presence of other reducing substances such as cysteine and glutathione.

However for the University of Louisville Medical School laboratory the indophenol method as modified by Wabnitz is quite accurate, rapid and reliable.

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C. DETERMINATION OF DIASTASE IN BLOOD AND URINE

INTRODUCTION AND HISTORY

Definition of Diastatic Activity: After the glucose content of the plasma is deducted from the total reducing substances, the difference represents the reducing power (in terms of glucose) of the reaction products formed from starch by the diastase content of 100 cc. of plasma. This figure is denoted as diastatic activity. Thus when one says that the diastase value of a sample of blood plasma is 120, it is meant that 100 cc. of that plasma produces from starch, cleavage products which have the same copper-reducing power as 120 mg. of glucose.

Normal diastatic activity is 80 to 150, with occasional extensions beyond either limit.

The problem of determining the diastatic activity of blood and urine has been much researched and discussed but the research workers do not agree upon the accuracy or clinical significance of the methods used. However, hormonal regulation of the amount of diastase in blood and tissues is proposed.

The accurate determination of diastatic activity in blood and urine is apparently of immediate forthcoming importance. According to Probstein, Wheeler and Gray (7) and Somogyi (10), diastase determinations may become an increasingly valuable aid in differential diagnosis. This growing value in clinical diagnosis caused interest in diastase to increase in recent years.

Diastase values of 80-150 mg./100 cc. blood or urine are found in persons who have no known disease. The values may be moderately raised in posterior perforated ulcers, near or at the pancreas and normal or subnormal in anterior perforated peptic

ulcers. Values are very high in acute pancreatitis. An increase in value may also be due to inflammatory conditions and duct occlusions of the pancreas. Diastase values are decreased in diabetics (below 80 mg.), which might be a sign of disturbed liver function. This subnormal diastase level may prove valuable in predicting abnormal liver functions when other tests failed to disclose anything. Obesity from over eating gives low values due to fatty infiltration or degeneration of the liver. Definite subnormal diastase values (as low as 20 mg.) are observed in ketosis, diabetic coma and preceding coma. The urinary diastase test is a useful index to the presence of acute pancreatic necrosis according to Smyth (8).

According to Chesley (2) results on diastatic activity vary due to different digestibility and varying proportions of alpha and beta amyloses and the dextrins.

THEORY AND METHODS

1. Micromethod for estimation of diastatic activity in human blood and urine, according to Michael Somogyi. (9). The method is based upon color reaction with iodine.

Selection of end point: The end point is that stage of the reaction at which the violet hue just disappears, yielding to the brownish-red color characterisitic of erythrodextrins. A 100 watt frosted bulb serves as the standard source of light and a clear, transparent starch solution was used which did not become turbid.

Object of Measurement: Time is the object of measurement. A single reaction mixture of standard starch content and of standard total volume is prepared from which periodically samples are withdrawn for testing with iodine until the end point has been reached. The time required is recorded.

Other factors: pH and electrolyte concentration are the same in amyloclastic process as in the saccharogenic reaction. Standard temperature, 40°C.

Proportionality: This is satisfied when the quantitative relationship between substrate and enzyme is such that the reaction reaches the end-point within 8 to 20 minutes. When adapting to microtechnique for the assay of blood plasma, no more than 1.0 cc. of plasma should be required. The adequate substrate for this is 3.0 mg. of starch.

Reagents: Preparation of starch. Wash 100 gm. of pure U.S.P. corn starch by frequent stirring for about one hour of its suspension in 1 liter of 0.05% NaCl. After sedimentation and de-

cantation, repeat washing with NaCl solution again. Spread out starch and allow to dry in air.

Starch paste. Rub up 15 to 20 gm. starch in a mortar with 100 cc. of water and pour suspension into 900 cc. of boiling water. Boil 1 minute with stirring. Add 10 cc. of 25% NaCl solution and immerse flask in boiling water bath for 30 minutes-cover mouth of flask with inverted beaker. Stand at room temperature for a day and let the starch separate out as a sediment. Remove supernatant fluid by syphoning. This dilute starch paste is the stock solution which, properly diluted with 0.25% NaCl solution, furnishes the substrate.

Extent of dilution. Starch content (0.4-0.6%) of stock solution. Into a large test tube (25 x 200 mm.) 5 cc. of fluid are introduced, 1 cc. of 3.6 N HCl added and tube closed with a 1 hole rubber stopper and glass tubing 2 ft. long. This is to serve as a reflux condenser. Reflux in a water bath for 2½ hours. Neutralize with NaOH using phenol red indicator, dilute to 100 cc. and determine the glucose content. This, multiplied by 0.9 equals the starch content of the stock solution and serves as the basis for dilution. Example. If the concentration is 0.428%, 75 cc. are measured into a 500 cc. graduated cylinder and diluted with 0.25% NaCl solution to 428 cc. Starch concentration in mg. x 75 cc. = cc. x 75 mg. i.e., dilute 75 cc. stock solution to a volume 100 x the starch content in % with 0.25% NaCl. The diluted solution then contains 75 mgs. of starch and 250 mg. of NaCl per 100 cc. and is used as starch substrate. Keep in flask with a 2 hole rubber stopper, syphon tube and air

inlet tube with cotton. Sterilize by heating 2 hours a day for 2 or 3 weeks. Fill syphon tube with reagent, close with pinch-cock and rubber bulb of alcohol. Starch solution contains 75mg. of starch plus 250 mg. of NaCl per 100 cc. of solution.

Iodine solution. 0.002 N aqueous iodine solution containing 2% KI. Prepare by diluting 10 cc. of 0.1 N aqueous iodine solution to 500 cc. with a 2% KI solution.

Analytical Procedure: Into a test tube (of 14 to 16 mm. diameter) introduce 4cc. of starch solution (3.0 mg. starch) and put tube in water bath of constant temperature. A few minutes later add 1 cc. blood plasma from pipette into a reagent tube and start stop watch. Mix thoroughly. While the mixture is incubating, measure 0.5 cc. portions of the iodine solution into small test tubes of uniform diameter. After 5 or 6 minutes withdraw a 0.5 cc. portion with a pipette and add to one of the iodine tubes for observation under a 100 watt bulb. Test other samples at various times until the red brown color of erythrodextrin is seen with barely a tint of purple. At this, the end point, the duration of the reaction is registered on the stop watch.

Calculation: Diastase $= \frac{K \times 1}{\text{time} \times \text{dilution}}$ K is 1600.

2. Simple determination of diastase in the blood, method of Chrometzka and Erlemann. (3).

Method: Draw 1.6 cc. of blood from the fasting subject with a 2 cc. syringe containing 0.4 cc. of sterile 1.5% NaF solution. Mix in a sterile tube and transfer 1.2 cc. to a mixture of 1.8 cc. N/15 phosphate buffer (pH 6.8) plus 5 cc. of sterile 1% starch solu-

tion in physiolical saline solution. Add a few drops of toluene, remove 0.1 cc. for determination of reducing power, incubate for 12 hours and again determine reducing power of 0.1 cc. by the Hagedorn-Jensen method. (5). Diastase is present only in the plasma and is inactivated in 1 hour at 70°.

3. New procedure for making starch solution to be used in Somogyi method (9), Wabnitz method.

Method: Make up fresh every time a determination is run. Dissolve 0.075 gm. soluble starch in 40 cc. boiling water. Add 50 cc. of 0.5% NaCl and dilute to 100 cc. with distilled H_2O in a volumetric flask.

4. Modified Wohlgemuth method, Smyth's method. (8).

Method: Requires only 45 minutes. Add 1 cc. of urine to 2 of 13 test tubes - to all but first add 1 cc. of 1% NaCl solution. Mix the second and add 1 cc. of mixture to 3. Repeat. Dilution of 1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024, 1/2048, 1/4096. To all ad 2 cc. of 0.1% starch solution. Place in a water bath at 38-40°C. for 30 minutes. Cool to retard hydrolysis and add a saturated solution of iodine drop by drop. The end point is the tube showing the first appearance of blue color. Calculation is made from dilution of tube immediately preceding it.

Calculation: $\frac{2 \text{ cc. (of starch)}}{1/8 \text{ (dilution of urine)}} = 16$. Normal = 4 to 32 units.

5. Blood amylase, method of Elman. (4).

This is the best test for pancreatic function according to Elman (4), and the viscosimeter method is recommended with the determination of the time required to effect a 20% reduction in viscosity

of a standard starch solution.

Method: Select a 5 cc. viscosimeter in which water takes 15 to 20 seconds and the starch solution 40 to 50 seconds to fall between the two graduation marks. Place 5 cc. of starch solution in the viscosimeter tube in a water bath at 37.5°C. and determine with a stop watch the time required to fall between the two graduation marks. Add 0.4 cc. plasma, mix by blowing bubbles of air through it and test again every few minutes until 20% reduction has occurred in the time. Record the time required for this reduction to be obtained. Express in amylase units (A.U.), a unit being the amount of plasma in cc. that will cause a 20% reduction in one hour, by substituting in the formula 60/T.V. T is the time in minutes and V, the volume of plasma used.

Normally with 0.4 cc. plasma, the 20% reduction occurs in about 30 minutes. The normal is given as 4.3 to 6.8 units. Obstruction of the pancreatic duct with greater absorption into the blood will increase the number of units, while atrophy of the acinin causes a decrease. Jaundice and renal insufficiency do not interfere with the test. Pregnancy has no effect on blood amylase. Oxalated plasma will keep in the refrigerator 24 hours.

Starch solution. Suspend 3 grams soluble starch (Lintner) in 70 cc. of cold distilled water, mix and boil. Cool and make to 100 cc. volume with M/15 phosphate buffer of pH 6.8. Autoclave 15 minutes at ten pounds' pressure. Filter. The solution can be kept sterile and the portion to be used is boiled again.

6. Method for diastatic activity according to Bray. (1).

"The diastatic activity is the per cent of the soluble starch used (10 mg.) which is transformed into reducing sugar (calculated as glucose) by the 2 cc. of blood used." It is one-fifth of the amount of reducing sugar formed by the amylase per 100 cc. of blood.

Method: Set up as follows:

	Tube 1 Test	Tube 2 Control
Blood	2 cc.	2 cc.
Water Put in water bath at 40oC. When tubes reach this temperature, add	7 cc.	8 cc.
Starch solution, 1% Mix and keep tubes in water bath at 40°C. exactly 15 minutes, then place in ice water immediately. Add	1 cc.	
Sodium tungstate, 10%	2 cc.	2 cc.
Sulphuric acid, 2/3 N Mix, and add	2 cc.	2 cc.
Starch solution, 1% Mix, and add		1 cc.
Water Mix. When chocolate colored, centrifuge and determine the blood sugar in each fluid.	6 cc.	5 cc.

Calculation: Subtract the blood sugar from the total sugar after the action of the blood amylase, each expressed in mg. per 100 cc., and divide by five. $\frac{\text{Tube I} - \text{Tube II}}{5}$ = D.A. (diastatic activity) Normal values are 15 to 20. The blood should be fresh, not over several hours old.

Folin-Wu method for blood sugar, according to Bray. (1).

Method: Put 2 cc. of clear blood filtrate into a Folin

blood sugar tube and 2 cc. of glucose standards I and II into two similar tubes. Add to each tube, 2 cc. alkaline copper solution and place the tubes in boiling water for six minutes. Cool in cold water-bath for two minutes. Add 2 cc. molybdate solution to each tube, and let stand until bubbling has ceased. Make up to 25 cc. mark with distilled water, stopper each tube and mix by inverting, compare in colorimeter. Set standard at 20 mm.

Calculations: $\frac{S}{R} \times 100 =$ mg./100 cc., if weaker standard I is used, $\frac{S}{R} \times 200 =$ mg/100 cc. if stroner standard II is used.

CONCLUSIONS

Since the accurate determination of diastatic activity in blood and urine is apparently of immediate forthcoming importance in clinical diagnosis, the various methods now in existent in the literature were reviewed, and the most widely acceptable method was then tested for accuracy and dependability. The outstanding source of error of the best obtainable method was found in the starch substrate used. The problem lies in the preparation of a suitable, standardized starch substrate. The present method for obtaining the best available substrate requires about one week for preparation and standardization. Soluble starch even specially prepared by Merck (according to Lintner) yields erroneous results which cannot be duplicated. Results obtained in this study were fully in accord with those found in the literature.

The best method of determining diastatic activity is the method of Somogyi. This method gives reproductible results but requires a week for preparation and standardization of the starch substrate.

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D. DETERMINATION OF SULFANILAMIDE AND DERIVATIVES IN BLOOD AND URINE

INTRODUCTION AND HISTORY

Since the introduction of sulfanilamide, (p-aminobenzene-sulfonamide) and allied substances and their proved efficiency, their popularity and use has grown rapidly. Consequently it has become necessary to find means whereby their concentrations in blood, urine and cerebrospinal fluid can be determined in order to form a guide to dosage.

The determination of the concentration of sulfanilamide and derivatives present in blood and urine of persons undergoing treatment for certain diseases is especially important. In treatment a high concentration of the drug is maintained in blood and tissues for several days. The concentration of the drug in tissues is dependent upon the concentration on blood. The concentration in the blood from a single dose of the drug depends upon five things: the dose per unit of body weight, the rate and completeness of absorption from the intestinal tract, the distribution ratio of the drug in the body, the efficiency of the kidneys in excreting the drug from the body, and the amount present in the body in the inactive or conjugated form. The first and the fourth factors are considered by most doctors to be the most important. (9).

Sulfanilamide concentrations in the blood, according to Alyea, Daniel, and Yates (1), are proportional to the renal function and to the dosage administered. Failure with sulfanilamide may not be due to lack of concentration in the blood. The fluid intake does not affect the blood concentration, but does affect the urine concentration of all three drugs: sodium-di-sulfanilamide, di-sulfanilamide, and sulfanilamide.

The sulfanilamide in man is excreted chiefly in the free form,

but partly in the conjugated form or acetylated derivative. Absorption is completerin about four hours. It is recommended that a determination be made four hours after the initial dose to see if the concentration is optimum (8-10 mg./100 cc.), and in 24 hours, to see if this concentration is being maintained.

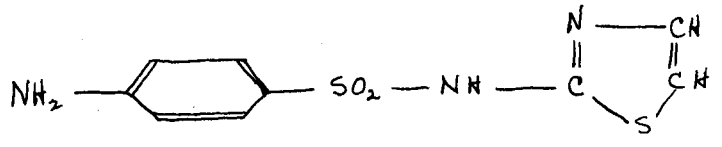
It requires 2 or 3 days to establish an equilibrium. Normally, most of the dye is excreted through the kidneys in 24 hours, but it requires about 2 or 3 days for the body to get rid of it completely. In renal impairment excretion is slow and the amount found in the blood may be much increased. Plasma contains about 10% more than whole blood. The drug is found in spinal fluid, saliva, pancreatic juice and bile in concentrations slightly less than in blood. It is found in all the secretions and body fluids. (9).

Not only does the effective blood concentration of sulanilamide vary with the severity of the disease, but also it varies with the type of disease. Dosage must be individualized and controlled by frequent quantitative blood determinations. Three things should be remembered: the method of administering the drug, the efficiency of the kidney in excreting it, and the effective blood concentration in various diseases. (This is determined only by free sulfanilamide since the conjugated form is therapeutically inactive). Absorption reaches a maximum in four hours. (11).

The test for sulfapyridine is highly important to physicians in estimating the bacteriostatic effect of sulfapyridine in the treatment of pneumococcic pneumonia and its complications. The concentration of sulfapyridine in the blood is determined by the rate of absorption,

the rate of excretion, and the rate of conversion. Absorption, excretion and conversion vary independently and are influenced by the unknown factors. Unless the parts per thousand in the blood or milligrams per hundred cubic centimeters are known, clinical failure cannot be ascribed either to inefficiency or to inadequate concentration of the drug. When the concentration of the drug is too low, larger and more frequent oral doses or parenteral administration may be employed.

Sulfathiazole (2-sulfanilylaminothiazole) is closely related chemically to sulfanilamide and sulfapyridine. Its empirical formula is $C_9H_9N_3O_2S_2$ and molecular wight is 225. The structural formula is:



The melting point is 200-202°C. Its taste is slightly sweet. It is poorly soluble in cold water; somewhat more soluble in hot water; but easily soluble in alkalis and alkaline earths with the formation of salts - such as the sodium salt which is stable in concentration up to 20%.

Sulfathiazole is the newest of the sulfanilamide derivatives and investigation reveals that it possesses bacteriostatic properties of advantage over the other sulfonamid derivatives. In combatting pneumococcic pneumonia and the more serious staphylococcic infections, sulfathiazole appears to exert prompt anti-bacterial properties. Its chief advantages in comparison with sulfapyridine and sulfanilamide are believed to be: more uniform absorption, less conjugation after absorption, less tendency to cause serious nausea or to provoke

vomiting , and greater effectiveness against the staphylococcus and the pneumococcous. In addition, clinical investigation and animal experimentation reveal that sulfathiazole has promise of a wider range of therapeutic usefulness.

Sulfathiazole has been used with satisfactory results in pneumococcal pneumonias and staphylococcal infections such as staphylococcic cellulitis, lymphangitis or acute osteomyelitis, and in treating large boils and carbuncles with accompanying toxic symptoms. Many other applications are suggested in articles of recent publication. Although successful therapeutci results are reported, their number still remains small. Much additional work and many more cases of similar nature should be reported upon before the drug is to be considered of specific value in these cases. For the sake of completeness, however, there is listed some of the work now in progress: Sulfathiazole is being investigated in the treatment of numerous pneumococcic and staphylococcic infections. It has a definite bacteriostatic effect on the organisms found in urinary infections, exerting a definite effect against the Streptococcus fecalis which had previously been found resistant to sulfanilamide. It should not be used in meningococcal meningitis since it is though to penetrate into the spinal fluid slowly and only to a limited extent. It is effective against Streptococcus hemolyticus and the virus of lymphogranuloma venereum, and more effective against the gonococci and the organisms of the colon-typhoid-dysentery group than either of the aforementioned derivatives.

These drugs should be carefully controlled in dosages be-

cause hepatic damage is not infrequent. With sulfathiazole therapy there has been greater freedom from severe nausea and vomiting than with sulfapyridine. Drug and fever rashes have been observed; cases of conjunctivitis, hematuria, anuria, leukopenia, and signs of renal irritation have been noted and described in sulfathiazole therapy as well as sulfapyridine and sulfanilamide therapy.

THEORY AND METHODS

The two most widely used methods of analysis are basedupon diazotization of the aminobenzene sulfonamid and coupling the resulting diazo compound in acid solution with dimethyl-a-naphthyl-amine or N-(1-Naphthyl)-ethylenediamine-dihydrochloride.

In the first method the diazotization was accomplished with nitrous acid and the coupling with dimethyl-a-naphthylamine in acid solution produced a purplish red azo dye which can easily be estimated by colorimetric comparison. This reaction depends upon the presence of an amino group substituted in the benzene ring and will estimate any compound to which the sulfonamide is changed in the organism and in which the amino group is intact. The color reaction is exceedingly delicate, being detectable in a solution of the sulfonamide of 1 part in 20 million parts of water. (9).

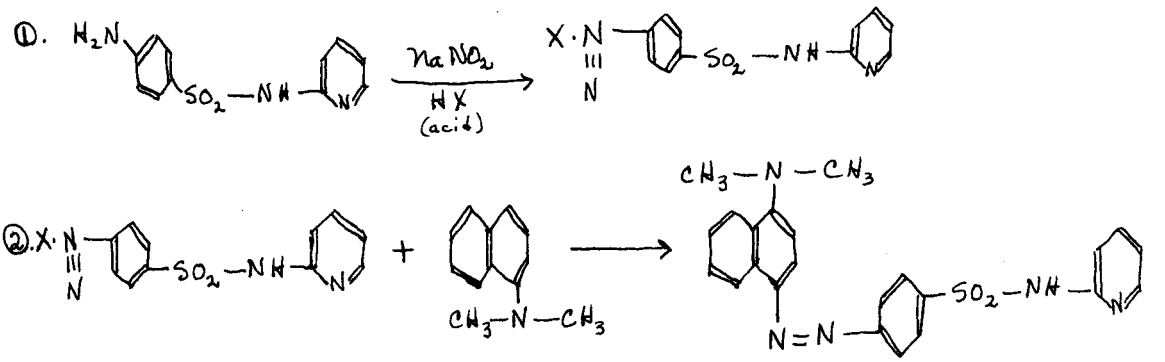
The use of N-(1-Naphthyl)-ethylenediamine dihydrochloride as the coupling agent offers the following advantages over other coupling compounds: Reproducibility, rapidity of coupling, greater sensitivity, elimination of buffer, and increased acid solubility of the azo dye formed by the coupling reaction. (3).

MacLachlan, Carey and Butler (7) described a good micromethod (modification of Marshall's method) using the Evelyn photoelectric colorimeter. It could not be adapted to use in the University of Louisville Medical School laboratory since the laboratory has no photoelectric colorimeter.

Another modification of Marshall, Emerson and Cutting's 1937 method which allows many estimations to be done with small quantities of material in short time was published by van der Zoo de Jong (18).

He uses phenol red and Na_2HPO_4 as standards which he claims will keep longer than those used by Marshall.

Higgins and Mendenhall (5) have determined a qualitative test for sulfapyridine in urinary calculi. In analysis of stones if positive nitrogen and sulfur tests are obtained the spot plate test should be used. A small amount of pulverized stone is placed in the depression of a spot plate and covered with 2 drops of 20% paratoluenesulfonic acid. Stand for 1 minute. One drop of freshly prepared 0.5% $NaNO_2$ (nitrite) added and let stand 1 minute and three drops dimethyl-a-naphthylamine added. Presence of sulfapyridine or derivatives will give a red or pink color which remains. Chemical reactions are:



Reaction involves diazotization of an aromatic amine with the subsequent coupling necessary to synthesize the colored compound. The reaction is not specific except that there are no such compounds in normal urine.

1. Micro-bedside test for the determination of sulfanilamide group concentration in body fluids, method of Schaeffel. (15).

Reagents: 1. Standards containing drugs of the various sulfanilamide groupings in progressive proportion from 0.5 to 15 mg./100 cc. of liquid. Amounts of 2.5, 5, 7.5, 10, and 15 mg./100 cc. are sufficient for reporting the concentration in body fluids. (Standard solution made up to pH of 5 with trichloracetic acid.) 2. HCl, 4 N. 3. Trichloracetic acid solution, 10%. 4. Sodium nitrite, 0.1% solution. 5. Ammonium sulfamate, 0.1% solution (contains 0.1 gram ammonium sulfamate/100 cc. distilled water). 6. Marshall's reagent, 0.4% strength (contains 0.4 grams of N(1-Naphthyl)ethylenediamine dihydrochloride in 100 cc. of distilled water, keep in dark brown bottle and only make up enough to last two days). 7. Filter paper (absorption strip paper for spot testing from Sargent and Company Laboratory Supply House, Chicago.)

Procedure: Place a drop of fluid to be examined (0.01 to 0.05 cc.) in the depression of a hanging drop slide. Add a 0.02 cc. of 4 N HCl and 0.02 cc. of 10% trichloracetic acid. Break up and stir thoroughly with glass thread (for total sulfanilamide heat over flame to near dryness and repeat). Add 0.03 cc. of 0.1% sodium nitrite and stir for 30 seconds (if sulfanilamide is over 20 mg./100 cc. use 0.05 cc.). Add 0.02 cc. of 0.1% ammonium sulfamate (for large amounts of sodium nitrite use more ammonium sulfamate). Add 0.02 cc. of 0.4% Marshall's reagent. Stir. Prepare standards. Good for 1 week in ice box away from light. Conveniently prepared by diluting a larger amount. Insert in the depression containing the now red solution a strip of filter paper and let liquid spread by capillary action over

one end of filter paper. (Precipitated proteins do not interfere.) Compare unknown with standards. Color charts may also be prepared to facilitate determinations at bedside. The final amount of liquid in depression should not exceed from 0.125 to 0.150 cc.

2. Determination of para-aminobenzenesulfonamide in urine and blood, method of Marshall, Emerson and Cutting. (9).

Reagents: N/10 HCl. 2. Sodium Nitrite, 01% (freshly prepared). 3. Ethyl alcohol (95%). 4. Dimethyl-a-naphthylamine, 1 cc. to 100 cc. of alcohol. 5. A standard solution of para-aminobenzenesulfonamide, 200 mg./liter. From this solution standard solutions containing 1.0, 0.5, and 0.2 mg. per hundred cc. can be prepared. The standard solution appears to keep unchanged for several months in icebox.

Procedure: Dilute urine to from 0.5 to 1.5 mg./100 cc. of sulfonamide. Measure ten cc. of this diluted urine into a small flask and add 2 cc. HCl, 1 cc. sodium nitrite, 5 cc. alcohol and 1 cc. dimethyl-a-naphthylamine. Shake. Treat ten cc. of an appropriate standard similarly. After a few minutes (5-10 minutes) compare in colorimeter with all glass cups.

Blood. Run 1 volume blood slowly with shaking into 9 volumes of alcohol, stopper flask and let stand 10 minutes or more. Filter mixture, measure 10 cc. of filtrate into flask and add 5 cc. water, 2 cc. HCl, 1 cc. sodium nitritie and 1 cc. dimethyl-a-naphthylamine. If colored solution is slightly turbid, let stand 5 minutes and filter. Use clear filtrate for colorimetric comparison. Prepare an appropriate standard at same time by adding 1 cc. of a standard solution of the sulfonamide to 9 cc. of alcohol and treat as for blood filtrate.

Wait about 15-20 minutes before comparing.

Since only 92% of the sulfonamide is recovered from blood by this procedure, the final result is divide by 0.92 to obtain the correct concentration in blood. If the dimethyl-a-naphthylamine is added about 3 minutes after the sodium nitrite a more intense color is obtained and the recovery is practically 100%. Duplicate determinations check within 2-3%.

Conjugated compound in urine: Heat equal volumes (usually 1 cc.) of urine and N HCl in a test tube (large) covered by a small beaker in boiling water for 30 minutes. Cool, add 1 drop 0.1% phenolphthalein, and neutralize with 2 N NaOH. Dilute to appropriate volume and determine as described for urine.

Conjugated compound in blood: Blood filtrates have been hydrolyzed by adding alcohol to 10 cc. of the filtrate, 2 cc. of 0.5 N HCl, evaporating alcohol by immersing in boiling water and heating for 30 minutes. This is as yet quite inaccurate.

3 Determination of sulfanilamide, method of Marshall and Litchfield. (10).

Procedure for blood: Measure 2 cc. of oxalated blood into a flask and lake with 14 cc. of 0.05% saponin solution. (Laking can also be accomplished by diluting with water at least 15 minutes before addition of the protein precipitant.) After laking is complete (1 or 2 minutes), add, with shaking, 4 cc. of p-toluenesulfonic acid solution (20 grams dissolved in water and diluted to 100 cc.) After 5 minutes filter the mixture and measure 10 cc. of the filtrate into a flask. Add one cc. of a freshly prepared 0.1% sodium nitrite solution

and shake. After 3 minutes add 1 cc. of 1 M sodium dihydrogen phosphate containing 0.5% ammonium sulfamate (prepared by dissolving 13.8 gms. $NaH_2PO_4.H_2O$ and 0.5 gms. ammonium sulfamate in 100 cc. of water) and after 2 minutes add from a burette 5 cc. of dimethyl-a-naphthylamine solution (1 cc. dissolved in 250 cc. of 95% ethyl alcohol). Treat ten cc. of the standard solution of sulfanilamide containing toluene-sulfonic acid (described below) like the blood filtrate and compared with the unknown. Make comparison 10 minutes after addition of the naphthylamine. A 1 mg.% standard is satisfactory for bloods containing from 5-20 mg% sulfanilamide. Higher concentration should be diluted.

Sulfanilamide standard solution: Dissolve 200 mg. in water and dilute to 1 liter. Keeps for several months on ice. Prepare standards in water of 1, 0.5, and 0.2 mg.% by diluting 5, 2.5, and 1 cc. of stock solution to 100 cc. To prepare standard solutions containing toluenesulfonic acid for use in the blood method, dilute 5, 2.5 and 1 cc. of the stock solution plus 18 cc. of the toluene-sulfonic acid solution are diluted with water to 100 cc.

Determination of the conjugated sulfanilamide: Dilute 2 cc. of blood with 30 cc. of saponin solution, and precipitate with 8 cc. of toluenesulfonic acid solution. Determine the free sulfanilamide in this filtrate as described above. To estimate the total sulfanil-amide, place 10 cc. of the filtrate in a test tube (15 x 150 mm.) graduated at 10 cc. and heat in a boiling water bath for 30 minutes. Allow the solution to cool and again make to 10 cc. Then repeat the procedure as above. The difference between the values obtained before and after hydrolysis gives the amount of conjugated compound.

Procedure for the urine: Dilute urine to contain about 1 mg.% of sulfanilamide. Add to 10 cc. of the diluted urine 1 cc. of tolunesulfonic acid solution and 1 cc. of 0.1% sodiun nitrite. After three minutes add 1 cc. of 1 M sodium dihydrogen phosphate containing 0.5% ammonium sulfamate followed in 2 minutes by the addition of 5 cc. of the dimethyl-a-naphthylamine solution. Treat a standard solution of sulfanilamide in water (usually 1 mg.%) in the same way and after 10 minutes compare the solutions.

To determine the acetyl derivative in urine, treat 1 cc. with 2 cc. of N HCl, heat in a boiling water bath for 30 minutes, allow to cool and neutralize with 2 N NaOH. Dilute the solution to appropriate volume (1:50 - 1:200) and use 10 cc. of the diluted solution for determining total sulfanilamide by the method described for urine.

Note: Trichloracetic acid can also be used in place of p-toluenesulfonic acid as the protein precipitant. Use one volume of a 15% solution of trichloracetic acid for each 4 volumes of diluted blood. The procedure for free sulfanilamide is the same as described above. To determine the total, treat 10 cc. of filtrate with 1 cc. of 2 N HCl, heat in a boiling water bath for one hour, cool and adjust the volume to 10 cc. The subsequent procedure is as above except that a 2 M phosphate buffer containing 0.5% ammonium sulfamate is used. Also the standard sulfanilamide solution used is prepared with trichloracetic acid instead of p-toluenesulfonic acid.

4. Determination of sulfanilamide, method of Bratton and Marshall. (3).

Reagents: 1. Trichloracetic acid: 15 gm. dissolved in water and diluted to 100 cc. 2. Sodium nitrite: 0.1 per cent solution.

3. N-(1-Naphthyl)ethylenediamine-dihydrochloride: An aqueous solution containing 100 mg. per 100 cc. (Keep in a dark colored bottle.) 4. Saponin: 0.5 gm. per liter. 5. Hydrochloric acid: 4 N. 6. Ammonium sulfamate: 0.5 gm. per 100 cc. 7. Sulfanilamide stock solution: 200 mg. per liter in water. (This solution can be kept for several months in the ice box. The most convenient standards to prepare from the stock solution are 1, 0.5, and 0.2 mg. per cent. To prepare these, 5, 2.5, and 1 cc. of the stock solution plus 18 cc. of the 15% solution of trichoracetic acid are diluted to 100 cc.)

Procedure for blood: Measure 2 cc. of oxalated blood into a flask and dilute with 30 cc. of saponin solution, and after 1 or 2 minutes precipitate with 8 cc. of the solution of trichloracetic acid. Determine the free sulfanilamide in the filtrate as follows: Add 1 cc. of the sodium nitrite solution to 10 cc. of the filtrate. After 3 minutes standing, add 1 cc. of the sulfamate solution and after 2 minutes standing add 1 cc. of the solution of N-(1-Naphthyl)-ethylene-diamine-dihydrochloride. Compare the unknown with an appropriate standard which has been treated as above. This comparison can be made immediately and no change in color is observed for 1 hour or more. To determine the total sulfanilamide, treat 10 cc. of the filtrate with 0.5 cc. of 4 N hydrochloric acid, heat in a boiling water bath for 1 hour, cool and adjust the volume to 10 cc. The subsequent procedure is as stated above for determining free sulfanilamide.

Procedure for urine: Dilute protein free urine to contain about 1 to 2 mg. per cent of sulfanilamide. Dilute 50 cc. of the diluted urine plus 5 cc. of the 4 N hydrochloric acid to 100 cc.

Treat 10 cc. of the product of this second dilution as a blood filtrate for free sulfanilamide, and heat 10 cc. without further addition of acid for total sulfanilamide. If the urine contains protein, dilute it and treat by the procedure for blood.

5. Determination of sulfanilamide (p-aminobenzenesulfonamide) in biological media, method of Scudi. (16).

Method: Use oxalated blood and precipitate blood protein by Folin-Wu method (modified by combining the tungstate and water prior to laking the blood. To 5 cc. of blood filtrate add 1 cc. of sodium nitrite (0.1%) and 1 cc. HCl (0.1 N) and stir. Let stand for 3 minutes and add 1cc. sodium carbonate (1.0%), (reaction must be neutral to litmus) and 1 cc. chromotropic acid (50 mg.%). Mix by inverting tube once and compare against appropriate standard (either 1 or 2 mg.%). Final value must be divided by 0.81 because the method is only 81% accurate.

Urine must not be preserved with formalin. Dilute urine 1:100 or 1:200 (final concentration 1 to 2 mg.% sulfanilamide). To 5 cc. diluted urine add 1 cc. sodium nitrite, 1 cc. HCl and shake and let stand for 3 minutes. Add 1 cc. sodium carbonate and 1 cc. chromotropic acid. Mix by inverting tube once and read against standard. Method is 87% accurate. Divide answer by 0.87.

6. Determination of para-aminobenzene sulfonamide (sulfanilamide) in urine and blood, Doble and Geiger modification of Marshall's method. (4).

Reagents: 0.1 N HCl, 0.1% solution of sodium nitrite (freshly prepared), 0.5% solution of diphenylamine in 95% alcohol, standard

solution of 200 mg. sulfanilamide/ liter of water, 95% alcohol.

Free sulfanilamide in urine: Dilute 1;50 (contains 0.5 to 1.5 mg. of sulfanilamide/100 cc.). To 10 cc. dilute urine add 2.0 cc. HCl, 1.0 cc. sodium nitrite and 5.0 cc. of alcohol. Shake, let stand 3-5 minutes and add 1.0 cc. diphenylamine. After 15 minutes compare with standard similarly treated.

Total sulfanilamide in urine. Heat for 30 minutes in a water bath equal volumes of urine and N HCl, cool, add 1 drop phenolphthalein, neutralize with 2 N NaOH. Dilute and treat as above. As much as 40% is in conjugated form.

Free sulfanilamide in blood: Let 1 volume of blood and 9 volumes of alcohol stand 15 minutes and filter. To 10.0 cc. of filtrate in flask, add 5 cc. water, 2.0 cc. 0.1 N HCL and 1.0 cc. sodium nitrite and shake. Let stand 3-5 minutes and add 1.0 cc. diphenylamine. If turbid, filter after 5 minutes. Prepare standard by adding 1.0 cc. sulfanilamide of suitable strength to 9.0 cc. alcohol and treating same as filtrate. Compare after 15 minutes.

Total sulfanilamide in blood: Let 10 cc. of filtrate and 5.0 cc. of 0.1 N HCl stand 30 minutes in boiling water bath with reflux condenser. Cool. Treat as above omitting addition of 5.0 cc. of H_2O 0.1 N HCl. Prepare standard as before except that no water is added and 5 cc. of 0.1 N HCl are used in place of 2 cc. The free standards will not give over 10% error.

7. Determination of sulfanilamide in blood, method of Kamlet, (6).

Reagents: 1. Trichloracetic acid - 5% solution: Dissolve

50 grams of trichloracetic acid in 800 cc. of water and ilute to 1 liter. 2. Sodium nitrite, 1% solution. Dissolve 10 grams of sodium nitrite in 800 cc. of distilled water and dilute to 1 liter. Keeps indefinitely in amber glass stoppered bottle. 3. Dimethyl-a-naphthylamine, 1% solution. Dissolve 1 cc. in 100 cc. 95% alcohol. 4. Ethyl alcohol, 95%. 5. Sulfanilamide standard solution. Dissolve 200 mg. sulfanilamide in 800 cc. boiling distilled water, cool, and dilute to 1 liter. 1 cc. of this solution contains 0.2 mg. of sulfanilamide.

Procedure: To 2 cc. of oxalated or citrated whole blood, add 18 cc. of 5% trichloracetic acid solution. Mix, let stand 10 minutes and filter. Pipette 10 cc. filtrate into test tube. To a similar tube, add 1 cc. of sulfanilamide, let stand, add 6 cc. trichloracetic acid solution and 3 cc. of 95% alcohol. To both add 0.1 cc. sodium nitrite, mix, let stand 3 minutes, add 1 cc. dimethyl-a-naphthylamine, shake, compare after 10 minutes.

Calculations: $\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 20 =$ mg. sulfanilamide per 100 cc. whole blood. Or: set unknown at 20.00 mm. and compare. Reading of standard = mg. sulfanilamide/100 cc. whole blood. 98.18% accurate with Marshall's 1937 method. (8).

8. Determination of sulfanilamide in tungstic acid blood filtrates by means of Na-β-naphthoquinone-4-sulfonate, Schmidt's method. (13).

Method: Use 5 cc. of oxalated blood to prepare Folin-Wu blood filtrates. Make a series of standards containing 0.02 to 0.2 mg. of sulfanilamide/10 cc. For convenience set up 5 standards cor-

responding to 2, 4, 7, 10, and 15 mg.% bloods and use the one the unknown most closely matches. To standards and unknown filtrates add 1 drop of 0.1 N HCl and 1 cc. of freshly prepared 0.05% sodium- -anphthoquinone-4-sulfonate, mix and place in dark for 45 to 60 minutes. Read in colorimeter against standard it most closely matches. (Standard set at 20.0 mm.)

9. Determination of sulfanilamide in cerebrospinal fluid by means of sodium-beta-anphthoquinone-4-sulfonate, Schmidt's method. (14).

Method in acid medium: Dilute 1 cc. spinal fluid to 10 cc. with distilled water. Measure into test tubes ten cc. quantities of various standards containing 0.01 to 0.20 mg.% sulfanilamide, equivalent to 1 to 20 mg.% fluids. Add 1 drop of 1.5 N HCl so pH of each solution will be from 2.0-2.5. This degree of acidity prevents any reaction between the reagent and the nitrogenous constituents normally present in the spinal fluid.

Add one cc. of freshly prepared 0.05% sodium-beta-naphthoquinone-4-sulfonate to each tube, mix contents and place in a dark place for 1 hour. The orange-red color shows poor proportionality, so the standard and unknown must be of approximately the same value, or concentration. It is generally necessary to prepare 2, 4, 7, 10, 15, and 20 mg.% standards. After the colors develop any two may be mixed should an intermediate be required. This method is much simpler but Marshall's has greater color intensity and proportionality.

10. Sulfanilamide determination in blood, method of Andrews and Strauss. (2).

Method: Add 2 cc. oxalated blood drop by drop from a pipette to 8 cc. 5% trichloracetic acid. Precipitate protein and filter leaving a clear filtrate. Next add 3 cc. of fourth normal NaOH, 4 cc. filtrate and 1 cc. Ehrlich's reagent. The yellow color develops immediately to maximum color intensity. Compare in colorimeter to the color developed by adding 1 cc. of the reagent to 7 cc. of a standard solution of sulfanilamide in distilled water, or diluting 1.25 cc. of the reagent to 10 cc. with standard. Prepare the standard containing 0.08 mg. of sulfanilamide per 7 cc. of water by dissolving 0.8 gm. sulfanilamide in 700 cc. water, or 1.143 gm. in 1000 cc. water and dilute 1:100.

If the unknown is set at 10 in the colorimeter, the readings of the standard will be mg./100 cc. blood. When concentration of unknown exceeded 10 mg. exact results were not obtained until the unknown was set at 5 and the readings of the standard multiplied by 2.

Reagents: 5% trichloracetic acid, Ehrlich's reagent (3 gm. para-dimethylaminobenzaldehyde added to 100 cc. water containing 3 cc. concentrated sulfuric acid) and fourth normal NaOH. All reagents and standard are stable.

For sulfapyridine : (equally satisfactory). A sulfapyridine standard may be made or the sulfanilamide standard used and the result multiplied by 1.5 to correct for the difference in molecular weight. This is a modification of Werner's method which saves half the time of Marshall's method. However, it has disadvantages at low concentrations and because of the use of yellow color in colorimetry.

11. A method for quantitative estimation of sulfanilamide and sulfapyridien in the blood and urine, Williams method. (19).

Reagents: 1. Ehrlich's reagent: Para-dimethylaminobenzaldehyde, 3.0 gm., concentrated sulfuric acid, 7.0 cc., and distilled water to make 100.0 cc. 2. Trichloracetic acid, 5.0 gm., and distilled water to make 100.0 cc. 3. Tenth normal NaOH. 4. 0.4 N NaOH. 5. Tenth normal HCl. 6. Sulfanilamide standard (1 cc. - 0.1 mg.), sulfanilamide, 0.1 gm., distilled water to make 100.0 cc., 7. Sulfapyridine standard (1 cc. - 0.05 mg.), sulfapyridine, 0.05 gm., distilled water to make 100.0 cc., 8. Lewis-Peebles colorimeter. 9. Lewis colorimeter tubes.

Methods: A. Free sulfanilamide in the blood: Prepare a protein free filtrate by adding 5 cc. of oxalated blood drop by drop to 20 cc. of trichloracetic acid. Shake and filter. (Tungstic acid is not suitable) Set up two Lewis colorimeter tubes and dose them as follows:

	Unknown	Standard
Blood filtrate	4.0 cc.	0
0.4 N NaOH	2.0 cc.	0
Sulfanilamide standard	0	0.8 cc.
Ehrlich's reagent	0.5 cc.	1.0 cc.
Distilled water to make	10.0 cc.	20.0 cc.

Mix and remove enough from standard tube to match color in unknown tube, read at once.

Calculation: Amount of fluid in standard tube divided by two equals the number of milligrams of sulfanilamide in 100 cc. blood.

B. Free sulfanilamide in urine: Dilute the urine 1:100 with distilled water.

	Unknown	Standard

Diluted urine	4.0 cc.	0
Sulfanilamide standard	0	0.8 cc.
Ehrlich's reagent	0.5 cc.	1.0 cc.
Distilled water to make	10.0cc.	20.0 cc.

Read at once.

Calculations: Reading of standard tube times ten gives the number of milligrams of sulfanilamide in 100 cc. of urine.

C. Free sulfapyridine in the blood: Filtrate prepared as above directed.

	Unknown	Standard
Blood filtrate	4.0 cc.	0
0.4 N NaOH	2.0 cc.	0
Sulfapyridine standard	0	1.6 cc.
Ehrlich's reagent	0.5 cc.	1.0 cc.
Distilled water to make	10.0 cc.	20.0 cc.

Read at once.

Calculations: Reading of the standard tube divided by two equals the number of milligrams of sulfapyridine in 100 cc. of blood.

D. Free sulfapyridine in the urine: Dilute urine 1:100 with distilled water.

	Unknown	Standard
Diluted urine	4.0 cc.	0
Sulfapyridine standard	0	1.6 cc.
Ehrlich's reagent	0.5 cc.	1.0 cc.
Distilled water to make	10.0 cc.	20.0 cc.

Read at once.

Calculations: Reading of the standard tube times ten equals the number of milligrams of sulfapyridine in 100 cc. of urine.

E. Total sulfanilamide in the blood: Prepare filtrate as outlined. Heat 2 cc. of this filtrate for 30 minutes in a boiling water bath. Cool.

	Unknown	Standard
Treated filtrate	2.0 cc.	0
0.4 N NaOH	1.0 cc.	0
Sulfanilamide standard	0	0.8 cc.
Ehrlich's reagent	0.5 cc.	1.0 cc.
Distilled water to make	10.0 cc.	20.0 cc.

Read at once.

Calculation: Reading of standard gives number of milligrams of sulfanilamide in 100 cc. of blood.

F. Total sulfanilamide in urine: To 1 cc. of urine add 2 cc. N HCl and heat in boiling water bath for 30 minutes. Then add 2 cc. N Naoh to neutralize the acid. Dilute to 100 cc. with distilled water.

	Unknown	Standard
Treated urine	2.0 cc.	0
Sulfanilamide standard	0	0.8 cc.
Ehrlich's reagent	0.5 cc.	1.0 cc.
Distilled water to make	10.0 cc.	20.0 cc.

Read at once.

Calculations: Reading of the standard tube times twenty equals the number of milligrams of sulfanilamide in 100 cc. of urine.

G. Total sulfapyridine in blood: Heat 2 cc. of filtrate in boiling water bath for 30 minutes. Cool.

	Unknown	Standard
Treated filtrate	2.0 cc.	0
N/4 NaOH	1.0 cc.	0
Sulfapyridine standard	0	1.6 cc.
Ehrlich's reagent	0.5 cc.	1.0 cc.
Distilled water to make	10.0 cc.	20.0 cc.

Read at once.

Calculation: Reading of the standard tube gives the number of milligrams of sulfapyridine in 100 cc. of blood.

H. Total sulfapyridine in the urine: To 1 cc. of urine add 2 cc. of N HCl and heat in a boiling water bath for thirty minutes. Then add 2 cc. of N NaOH and make up to 100 cc. with distilled water.

	Unknown	Standard
Treated urine	2.0 cc.	0
Sulfapyridine standard	0	1.6 cc.
Ehrlich's reagent	0.5 cc.	1.0 cc.
Distilled water to make	10.0 cc.	20.0 cc.

Read at once.

Calculation: Reading of the standard tube times twenty equals number of milligrams of sulfapyridine in 100 cc. of urine. About one-half is present in free form and one-half in conjugated form. To find total sulfanilamide, free sulfanilamide times two equals total sulfanilamide.

12. Determination of sulfanilamide in Urine, Wabnitz method.

Preparation of urine sample: Dissolve 0.2750 grams of sulfanil-

amide in 250 cc. urine. $\frac{275 \text{ mg.}}{x \text{ mg.}} = \frac{250 \text{ cc.}}{100 \text{ cc.}}$ 110 mg./100 cc. undiluted urine.

Preparation of standard: 0.1 mg./10 cc. (sulfanilamide dissolved in water.)

Procedure for the determination of sulfanilamide in urine: Dilute 1 cc. urine sample to 100 cc. (with distilled water in a 100 cc. volumetric flask. Treat 10 cc. dilute urine and 10 cc. standard solution in a 150 cc. Erlenmeyer flask simultaneously as follows: Add: 2 cc. 0.1 N HCl - shake well, 1 cc. dimethyl-alpha-naphthylamine solution. Shake and compare in colorimeter.

Calculation: Mg. sulfanilamide per 100 cc. of urine equals reading of standard divided by reading of unknown times 100.

13, Bedside test for sulfapyridine, Ratish and Bullowa modification of Marshall's method. (12).

Reagents: 1. Ether, 2. 15 percent trichloracetic acid solution, 3. 0.1 per cent solution of sodium nitrite, 4. 1 per cent solution of urea, 5. A solution of alpha-dimethylanaphthylamine containing 1 cc. in 250 cc. of 95 per cent ethyl alcohol. (this reagent should be kept in a dark dropping bottle.)

Apparatus: 1.2 cc. Luer syringe and needle, 2. 1 test tube of 20 cc. capacity with round bottom, stoppered with cork and marked at 1 cc. and 6 cc., 1 centrifuge tube with long taper, graduated to 15 cc. in 0.1 cc. divisions, fitted with rubber s topper, 3. 5 dropping bottles to contain the reagents listed above, 4. a comparator block 2.5 inches by 4.5 inches, with 4 openings to take test tubes 100 by 12 mm., and color s tandards.

Method: Into a Luer syringe draw approximately 1.5 cc. of venuous blood. Invert the syringe with the needle still attached and deliver the blood, drop by drop, into the round-bottomed test tube, to the 1 cc. mark. Insert the stopper and shake vigorously for two minutes. The fluids will rapidly separate into two layers, with the ethereal extract of sulfapyridine in the upper layer. Slowly release the stopper. Cautiously decant the ethereal extract into the centrifuge tube to the 0.5 cc. mark and set the extraction tube aside for duplicate tests.

By means of a dropping bottle add 15 per cent trichloracetic acid solution to the 5 cc. mark, place the rubber stopper over the mouth of the tube and shake vigorously for ten to twenty seconds. Add 0.5 cc. of 0.1 per cent sodium nitrite solution (7 to 8 drops from a dropping bottle), bringing the solution to the 5.5 cc. mark. Again shake vigorously for twenty seconds, add 0.5 cc. of 1 per cent urea solution dropwise from a dropping bottle. Finally, add alpha-dimethyl-naphthylamine to the 8.5 cc. mark from its dropping bottle, close the tube with the rubber stopper, and invert once or twice. The white opalescence is soon replaced by a purplish red color. After five minutes the color is fully developed and the tube may be compared with standards in the comparator lock. All values of this ether extraction method lie withinn75 to 95 per cent of those obtained by the Marshall method, using a photocolorimeter.

The quantitative estimation of sulfapyridine depends upon the formation of a purplish red dye. This diazotization may be completed and the full color developed in five minutes. The rate of

color formation after coupling with alpha-dimethylnaphthylamine, the comparative action of ammonium sulfamate and urea on the destruction of the excess nitrite, and the value of the trichloracetic acid and paratoluenesulfonic acid as media for the reaction were studied. With a photoelectric colorimeter results were obtained which indicated that either group of reagents may be used. Urea and trichloracetic acid are recommended because they are cheaper and more readily obtainable. While ether does not extract sulfapyridine from watery solutions of urine as efficiently as it does from blood, it extracts sulfapyridine from cerebrospinal fluid.

Preparation of color standards: Color standards may be readily prepared using phenol red. They are calibrated so that the value of sulfapyridine obtained from the sample corresponds to the true value. Dissolve 0.0075 gm. of phenol red in 100 cc. distilled water. To a series of 5 tubes, add 3.9 cc. of fifteenth molar potassium dihydrogen phosphate and 6.1 cc. fifteenth molar secondary sodium phosphate. Then follow with 0.2 cc. of normal sodium hydroxide and phenol red as indicated. The volume of the phenol red should increase progressively in the different test tubes. The phenol red solutions are transferred to test tubes 100 by 12 mm. and sealed. The value of each tube should be marked on or permanently attached to it. The standards should be protected from direct sunlight. New standards should be prepared every six months.

14. Estimation of sulfathiazole in blood serum and urine, Sunderman and Pepper modification of Marshall and Litchfield's method. (17).

Procedure of analysis: Since the recovery of sulfathiazole from serum is within approximately 3 per cent of the theoretical, we have preferred routinely to perform our analyses on serum and to make an arbitrary correction for the losses encountered. The method that we have finally developed for the analysis of sulfathiazole in serum is essentially an adaptation of the Marshall and Litchfield procedure for the determination of sulfanilamide in whole blood. Run 2 cc. of serum (in blood serum containing high concentrations of sulfathiazole, 1 cc. of serum is employed) obtained from centrifuged clotted blood into a 25 cc. volumetric flask containing 10 cc. of 8 per cent trichloracetic acid. After shaking thoroughly to keep the precipitate finally divided, dilute the solution to the mark with distilled water. Mix the contents and allow to stand for 20 minutes; then filter through Whatman No. 44 paper to obtain a clear, colorless filtrate. Diazotize ten milliliters of the filtrate at room temperature with 1 cc. of a 0.1 per cent freshly made solution of sodium nitrite. Treat the solution after standing for 3 minutes with 1 cc. of a 0.5 per cent solution of ammonium sufamate. After mixing thoroughly, add 5 cc. of dimethyl-alpha-naphthylamine (Solution contains 1 cc. dimethyl-alpha-naphthylamine in 250 cc. of 95% alcohol) coupling reagent; stopper the tube invert once and allow to stand for 10 minutes.

Comparisons of color development are made by means of a colorimeter (or photoelectric cell device) utilizing sulfathiazole standards of appropriate concentrations carried through the same procedures as with the unknown. It is important that the solutions be well shaken before placing them in the colorimeter cups to eliminate

small gas bubbles that may be present. (the gas is presumably nitrogen formed by the destruction of HNO_2 with $NH_2.SO_3.NH_4$.) Use a colorimeter (Bausch and Lomb) having an attachable lamp. Fit the eyepiece of the colorimeter with a No. 74 Wratten filter. Make standards of appropriate concentrations from stock solution of sulfathiazole containing 200 mg. per liter. It is convenient to have prepared standards having concentration of sulfathiazole of 0.2, 0.4, and 0.8 mg. per 100 ml., respectively.

Calculation: $\frac{\text{Reading of standard}}{\text{Reading of unknown}}$ x mg. of sulfathiazole in standard x dilution factor x correction factor = mg. per 100 ml.

Correction factor = 103.

Recovery of sulfathiazole from protein-free urine is practically quantitative. The procedure for serum may be applied to urine determinations disregarding the correction factor. It is preferable that appropriate dilutions of urine be made so that sulfathiazole concentration in the diluted urine is between 5 and 30 mg. per 100 cc. So far as analysis of urine is concerned, a number of writers have called attention to the great danger in the use of the drug by the occurrence of the crystallin form in the urinary passages. Accordingly, they studied the excretion of the drug inurine, its solubility, and crystalline structure. They found that both sulfathiazole and its acetyl derivative were about twice as soluble in urine of pH 7.6 as in urine of pH 5.6, hence it may be inferred that when crystalline concentrations owing to sulfathiazole therapy threaten, an effort should be made to keep the urine alkaline and to secure a large urinary volume.

15. Determination of sulfathiazole in blood, modified method of Bratton and Marshall. (3).

Reagents: 1. Trichloracetic acid solution containing 15 grams dissolved in water and diluted to 100 cc., 2. 0.1 per cent sodium nitrite solution (freshly prepared each week), 3. An aqueous solution of N-(1-Naphthyl)-ethylenediamine dihydrochloride. 100 mg. per 100 cc. (keep this solution in a dark colored bottle). 4. Saponin solution containing 0.5 gram per liter. 5. 4 N hydrochloric acid. 6. A solution of ammonium sulfamate, containing 0.5 gram per 100 cc. 7. A stock solution of sulfathiazole in water containing 200 mg. per liter. This solution is stable. The dilutions necessary as standards for the colorimetric comparisons have to be prepared freshly each time. The three standards are made to contain 1.0, 0.5, and 0.2 mg. per 100 cc. To make the first concentration use 5.0 cc., for the second, 2.5, and for the third, 10 cc. of the stock solution of sulfathiazole and add 18 cc. of the trichloracetic acid. Dilute all three to 100 cc.

Procedure: Withdraw 2 to 3 cc. of fluid, shake it with a few crystals of oxalic acid, transfer 2 cc. into a flask and dilute with 30 cc. of the saponin solution. After 1 or 2 minutes precipitate the blood proteins by adding 8 cc. of the trichloracetic acid solution, allow to stand five minutes and filter. To 10 cc. of the filtrate add 1 cc. of the sodium nitrite solution, after shaking and standing 3 minutes add 1 cc. of the sulfamate solution. After another 2 minutes add 1 cc. of the solution N-(1-naphthyl)-ethylenediamine hydrochloride. Compare the color developing in this mixture with the three standard

solutions within one hour. Treat the standard solutions as follows: Submite 10 cc. of each standard solution (as described above) to the same procedure, beginning with the addition of 1 cc. sodium nitrite, followed by the addition of 1 cc. naphthyl-ethylenediamine hydrochloride, et.

Determine the total amount of sulfathiazole in the blood (free plus acetylated thiazole) by heating in a boiling water bath another 10 cc. of the blood-trichloracetic acid filtrate for one hour, after having added 0.5 cc. of the 4 N hydrochloric acid; cool this solution, bring up to 10 cc. and treat as described above, starting again by adding 1 cc. of the sodium nitrite solution.

A rough estimation is made by comparing in a comparator the colors for the unknown with the colors of the standards. For more accurate estimations, special colorimeters may be used. While the above mentioned method can be performed in the physician's laboratory, for more accurate determinations it is advised that commercial or hospital laboratories be used.

CONCLUSIONS

In reviewing the recent literature sulfanilamide, sulfapyridine, and sulfathiazole determinations it was concluded that the method of Bratton and Marshall (3) for sulfanilamide usin N-(1-Naphthyl)-ethylenediamine dihydrochloride was the most widely accepted. The same method can be adapted to other compounds of the same family. The standard is prepared from the drug to be determined. This method of Bratton and Marshall (3) may be applied to the determination of sulfapyridine, sulfathiazole, and sulfanilamide derivatives by substituting the proper standard and carrying out the same procedure. For sulfapyridine, solutions of which are unstable, a sulphanilamide standard can be used.

Since the chromogenic effect of sulphapyridine is less than that of sulfanilamide, the formula should be changed when the sulfapyridine is determined with a sulfanilamide standard. The sulfanilamide standards can be used for most of its derivatives with a correction factor that must be determined for each drug.

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E. DETERMINATION OF LEAD IN BLOOD AND URINE

INTRODUCTION AND HISTORY

Lead is present in normal people in most tissues, especially in the bones. It is absorbed through the respiratory and gastro-intestinal tracts and sometimes through the skin. The source of lead in normal persons is food, water and dust particles. The average diet contains 0.2-0.35 mg. of lead per day. A normal person excretes 0.25-0.3 mg. per day, or about 0.04 mg./l. of urine. The calcium content of the diet and body effect the individual tolerance to lead. According to Chalmers (1), the normal lead content of blood varies from 30-90 micrograms of Pb/100 cc. blood. Lead may be found to the extent of 50 mg. or so in the urine of healthy individuals. It is increased in lead poisoning. Lead poisoning produces large black granules in apparently normal staining blood cells.

THEORY AND METHODS

1. The determination of Lead in biological materials, Thompsett method. (11). There are three steps: (1) Destruction of organic material, (2) Extraction of Pb with ether as a complex with Na-diethyldithiocarbamate; (3) Colorimetric estimation of lead with dithizone.

Reagents: 1. Con. HCl, 2. Con. HNO_3. 3. Perchloric acid. 4. Glacial acetic acid. 5. Ammonia (sp. gr. 0.88). 6. Ether. 7. Carbon tetrachloride. 8. 10% Potassium cyanide, diluted 1 in 10 as required. 9. 5% sulfurous acid, lead free. 10. 20% sodium citrate, lead free. To 1 liter of a 20% solution in water, add 100 ml. of 0.1% diphenylthiocarbazone in chloroform, shake and filter to remove small particles of chloroform. 11. 2% sodium diethyldithiocarbamate, before using shake up small volume with ether to remove traces of lead. 12. Standard solution of lead acetate. Dissolve 0.1831 gm. lead acetate ($Pb(C_2H_3O_2)_2.3H_2O$) in distilled water containing 5 milliliters of glacial acetic acid. Make up volume to 1 liter with distilled water. One milliliter is equivalent to 0.10 mg. of lead. Clean everything with hot dilute HCl and distilled water before use. 13. Dithizone (diphenylthiocarbazone), a 0.1% solution of crude dithizone in CCl_4 was prepared. Just before use a small volume was shaken with equal volumes of 0.5% NH_3. Dithizone passes into water layer. Let settle, separate and use ammoniacal extract directly.

Method: A. Extraction of Lead from urine: Evaporate 500 ml. of urine to dryness in a silica dish in a hot air oven. Ash residue by ignition over bunsen flame in a fume cupboard. Dissolve the ash in 100 ml. of water containing 5 ml. of concentrated HCl. Transfer the solution to a separating funnel, add 50 ml. of 20% Na-diethyl-

dithiocarbamate and extract the mixture with three 25 ml. portions of ether. Wash the ether extracts separately with water and transfer to a hard glass round-bottomed flask. Evaporate the ether and digest the residue with 1 ml. of concentrated sulfuric acid and 1 ml. of perchloric acid to destroy organic matter. Dilute the residue with water, 1 ml. of glacial acetic acid and 5 ml. of ammonia (0.88 sp. gr.) and dilute the mixture to 25 ml. with water.

For blood. To 100 ml. of lead free 10% Na_2HPO_4 in a silica dish add 20 ml. of blood. Dry in an oven and ash. Dissolve the ash in 50 ml. of water containing 5 ml. of concentrated HCl. Transfer to a separatory funnel, cool, add 5 ml. of 20% sodium citrate and make the solution alkaline to litmus with ammonia. Add 5 ml. of 10% KCn and 2 ml. of 2% Na-diethyldithiocarbamate. Extract twice with ether, using 20 ml. portions and wash each with water and put in a 100 ml. hard glass round bottom flask. Evaporate the ether, digest with 0.2 ml. of concentrated sulfuric acid, 0.5 ml. of perchloric acid, 3.5 ml. of water and 0.2 ml. of glacial acetic acid and 1.5 ml. of ammonia (sp. gr. 0.88).

B. Colorimetric estimation of lead: Preparation of standard: Dilute 1 ml. of concentrated sulfuric acid and a little water, 1 ml. glacial acetic acid, and 5 ml. of ammonia to 25 ml. with water giving ammonium acetate. Also add a known amount of lead to 5 ml. of this mixture. Also add 5 ml. of 1% KCn and 10 ml. of CCl_4. To this drop by drop add an excess of dithizone reagent, avoiding too great an excess. A sufficient excess has been added when CCl_4 layer reached the maximum degree of redness and the water layer is brown. Remove and

discard the water layer. Shake the CCl_4 layer with 5 ml. of 1% KCn until the water layers are no longer colored. This will require several portions of KCN. The CCl_4 layer may be passed through a filter to remove droplets of water and is then ready for comparison. The standard contains 0.02 mg. of lead if 2 ml. of a standard solution of lead acetate containing 0.01 mg. of lead per ml. are used.

Preparation of unknown urine: The extracted lead is contained as $PbSO_4$ in a solution of ammoniacal ammonium acetate having a volume of 25 ml.. For the estimation use 5 ml. of this solution, 5 ml. of 1% KCN and 10 ml. of CCl_4. The color of the solution is then developed as described above. If the lead content is low, use 10 ml. and the same amounts of the other reagents . If the lead is high, use a smaller volume than 5 ml. and dilute to 5 ml. with ammoniacal ammonium acetate. Develop the color in glass stoppered tubes.

For blood. Use the whole of the lead containing solution. To the mixture containing the lead in the flask used for the digestion add 5 ml. of 1% KCN and 10 ml. of CCl_4. Develope the color as before.

C. Blank test: In estimating the blank, the complete process should be followed through. Before colorimetric estimation, add 0.02 mg. of lead to the blank. Compare this, after the development of the color with dithizone, with a standard containing 0.02 mg. of lead. Then calculate the blank from the difference.

2. The determination of minute amounts of lead in urine, method of Kench. (5).

Reagents: Use pyrex glassware and glass distilled water throughout. Purify other reagents as follows: 1. Shake 2 N sulfuric

acid with CCl_4 - ether mixture (1:20) and 20 mg. of diethyldithiocarbamate and run off. Then shake the acid with ether alone and remove the latter by careful warming on a water bath. 2. Delead 20% sodium citrate and ammonia in a similar manner. 3. Obtain Merck's perhydrol and 10% KCN lead free. 4. Dissolve 5 mg. dithizone in 5 ml. of CCl_4 and shake with 5% NH_4OH. Acidify the aqueous layer with N HCl and take up the dye in CCl_4, leaving traces of lead in the aqueous layer. Finally, extract the purified dithizone with 5% ammonia just before use.

Method: Evaporate 100 ml. of urine with 100 ml. of 2 N sulfuric acid in a 350 ml. Kjeldahl flask and digest with the addition of 4 drops of perhydrol until clear. Dilute the digest with water, add 5 ml. of 20% sodium citrate and make the solution alkaline to litmus with ammonia (sp.gr. 0.88). After the addition of 5 ml. of 10% KCN, remove lead by shaking twice with ether and approximately 20 mg. of Na-diethyldithiocarbamate. Wash the ether extract with water, evaporate the solvent and digest the residue with 1 ml. of sulfuric acid. Add 3-4 drops of perhydrol and heat the solution for 5 minutes after clearing. Dilute the acid with 4 ml. of water and 0.2 ml. of acetic acid and add 3 ml. of ammonia (sp. gr. 0.88) to make the reaction alkaline. Wash out this solution into a 50 ml. separatory funnel (with very short stem) with 5 ml. of 1% KCN, and add 5 ml. of CCl_4. Shake the solution with a slight excess of dithizone solution (2-3 drops), draw off the water phase with a Pasteur pipette and wash out the remaining dithizone with 1% KCN. Run off the colored CCl_4 layer and compare with a standard (lead acetate) in a Klett microcolorimeter,

using photometric technique. Normal urine contains from 0.038 to 0.080 mg./liter, the average is 0.057 mg./liter.

3. The detection and estimation of small amounts of lead using a-diphenylcarbazide, method of Krans and Ficklen. (7).

If the sample is in solid form, it should be pulverized to 100 mesh, a representative sample taken and placed in 50 cc. of distilled water. The sample should contain not more than 0.2 mg. of lead. In the case of a lead bearing liquid or a suspension of lead bearing material, an aliquot portion containing about 0.2mg. of lead or less, should be used. Add to the sample 25 cc. of nitric acid (1:1), evaporate to between 2 and 4 cc., cool, and dilute to 80 cc. with distilled water. Add 4 drops phenolphthalein (0.5% in 1% sodium hydroxide) and neutralize with 25% sodium hydroxide, adding an excess of 5 drops. Acidify with 5% acetic acid until the pink color just disappears, and add 2 cc. in excess. Bring to the boiling point and precipitate the lead by adding 1 cc. of 1% potassium chromate. Place on a steam bath for one hour and allow to stand at about 20°C. overnight. Heat just below boiling for 15-20 minutes and filter hot through a filter (No. 42 Whatman - 11 cm.) which has been washed down with hot water. Rinse the beaker thoroughly onto the filter paper with hot water, washin the precipitate well with hot water in order to remove all traces of soluble chromates. Place 15 cc. of hydrochloric acid (1:2) in the precipitation beaker and rinse the sides well with the acid. Place the funnel containing the filter and precipitate over a 200 cc. volumetric flask and carefully pour the hydrochloric acid in the beaker over the entire surface of the filter paper. Rinse the beaker onto the filter paper and then

wash the filter thoroughly, using cold distilled water for both operations. To the solution add 2 cc. of s-diphenylcarbazide solution (1% in glacial acetic acid) and make up to the mark with distilled water. Shake well and with colorimeter estimate the amount of lead in the sample by comparing the intensity of the pink color with a known standard.

To make the lead standard for matching against the unknown, dissolve 0.142 gm. of potassium dichromate ($K_2Cr_2O_7$) in distilled water and dilute to 1 liter; 1 cc. of this solution is equivalent to 0.2 mg. of lead. Take 5 cc. of this solution, add 2 cc. of s-diphenylcarbazide, and make up to 1 liter; 1 cc. of this solution is equivalent to 0.001 mg. of lead.

All apparatus should be washed before using with hot 10% nitric acid, and then with distilled water to insure freedom from lead. A blank test should be run to determine the purity of the reagents. If the color standard is made up to the strength outlined, the sharpest color change is noted. A Campbell-Hurley modification of the Kennicott-Sargent colorimeter should be used. It is advisable to make up the s-diphenylcarbazide reagent and the color standard fresh each day. The stock solution of potassium dichromate may be kept indefinitely.

4. The chromate method for lead, Fairhall's method. (3).

Before analysis, tissues and feces must be freed from water by baking, i.e., heating in porcelain dish until it starts to char, and then ashed at dull red heat. Cool, extract with dilute hydrochloric acid and tartaric acid which dissolves lead phosphate.

Neutralize with sodium hydroxide. Acidify with hydrochloric acid until just acid to methyl orange. Place in ice bath and saturate

with hydrogen sulphide, and neutralise to phenolphthalein with sodium hydroxide. Acidify with acetic acid and add 2-3 drops of saturated solution of potassium chromate. A precipitate due to lead may be seen at this point; but can be hastened by boiling. If no turbidity, let stand overnight. Centrifuge, and wash all free chromate from the precipitate, dissolve the lead chromate in a little dilute hydrochloric acid, add an excess of potassium iodide solution and titrate the free iodine with 0.005 N sodium thiosulphate solution, using a drop of starch indicator. One cc. of 0.005 N sodium thiosulphate solution is equivalent to 0.3541 mg. of metallic lead.

For urine, make alkaline with ammonia and allow phosphates to settle out, entraining the lead phosphate (1 to 24 hours). Filter and ash the filter with the precipitate, or, centrifuge and ash the precipitate.

CONCLUSIONS

The various methods reviewed in the literature for the quantitative estimation of lead are time consuming and involve elaborate methods of developing the color for comparison with a standard. The different methods use Na-diethyldithiocarbamate in extracting the lead, s-diphenylcarbazide, and potassium chromate.

These methods have not yet been tested because the required reagents were not available.

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F. USE OF COLLOIDAL SOLUTIONS IN EXAMINING CEREBROSPINAL FLUID

INTRODUCTION AND HISTORY

On searching the literature it appeared the first recorded preparation of colloidal gold solution, or gold sol, was made by Faraday in 1857. His method was the reduction of a solution of gold chloride by phosphorus dissolved in ether. In 1897 Brady prepared a gold sol by passing an arc between gold electrodes under water. In 1898 Zsigmondy reduced gold chloride in a solution made alkaline with potassium carbonate, with formaldehyde. He also discovered that flocculation of gold particles with an electrolyted, such as NaCl can be prevented by specific protein concentrations. Lange adopted this phenomenon to spinal fluids. In 1912 he discovered that in a suitable saline concentration those fluid with syphilitic involvement cause flocculation of gold, while normal spinal fluids do not. He worked out the color ratings, scale of dilutions, and form of reporting which is still used in distinguishing the several types of syphilitic affections of the central nervous system. (11).

In the years from 1913 to 1923 there were various modifications of Zsigmondy's method for colloidal gold. Those most often used today are of three general types: The Eike modification (1913) using glucose in place of formaldehyde; the Miller modification (1918) using oxalic acid and formaldehyde; and the new Mellanby method (1923) using potassium oxalate. The glucose method yields beautiful preparations but they are not as sensitive as those produced by the Miller method. The Mellanby method is not suitable for preparation of large quantities. (11).

Satisfactory preparations will have the following characteristics: By transmitted light the color is rose red, cherry, or salmon red with no trace of blue tint. By reflected light the color is clear

brown. The pH should be 6 or 7. One and seven tenths volumes of 1% NaCl will precipitate 5 volumes of the colloidal gold overnight and generally in an hour. Numerous factors affect the preparation of a gold sol; the various observers are in complete disagreement as to their relative importance and as to wheter a given fador is favorable or to the contrary. (11).

Many difficulties of the usual methods of preparing gold sols for use in the Lange test may be ascribed to lack of reproducibility of the reagents. Some potassium carbonate absorbs water the amount depending upon the length of exposure to moist air. The crystals of gold chloride contain a variable amount of adsorbed water. The formaldehyde solutions often vary in strength and acidity. (8).

Difficulties may be eliminated by using a stable crystalline salt such as neutral potassium oxalate as the reducing agent, standardising to account for the variations in the gold chloride, and using a standard alkali for regulation of pH and a standard acid for final adjustment to the required sensitivity. (8).

According to Glasoe and Sorum (6) the results of syphilitic and tabetic fluids indicate that in the cases of paresis and syphilis the sensitivity of gold sols in the Lange spinal fluid test increases as the particle size increases and decreases as the pH indreases. The results on tabetic fluid indicate that these fluids respond to change in pH and particle size in gold sols. Paretic and syphilitic fluids act in the same fashion.

The Lange test for the clinical diagnosos of certain diseases of the central nervous system such as paresis, tabes, syphilis,

encephalitis, poliomyelitis, and meningitis, is not as widely used as its potential possibilities would seem to warrant. This limited use is doubtless due, in part, to the fact that gold sols, of the exact quality demanded by the test, are extremely difficult to prepare. Furthermore, so little is known definitely about the mechanism of the test and the specific role played by each of the reactants that irregularities are not readily interpreted.

The Lange test is regarded as a definite aid in diagnosis and as a valuable means of following the progress of several of the above mentioned diseases of the central nervous system.

The results obtained by Johnson, Aude, and Sorum (7) on the use of the photelometer in making Lange test readings indicate that the photelometer is a very sensitive and accurate method. It use enables the less experienced operator to make fine distinctions of color such as are not possible with visual readings. With it detections of even slight differences in color of gold sols are possible, thereby making it a valuable tool in the Lange test. However, the photelometer has no advantage over the visual method in practical clinical work, where the general forms of the curve and the relative colors of the solutions are more important than the actual colors.

The Lange test itself has a very interesting history; it was discovered quite by accident. Zsigmondy had produced colloidal gold sol and stabilized it against precipitation with NaCl solution by egg albumin solution. In 1912 Lange repeated the work of Zsigmondy and experimented with the highly albuminous cerebro-spinal fluid from a patient suffering from general paralysis of the insane. The sol,

to Lange's surprise was precipitated. As a result of his further investigations he discovered that cerebro-spinal fluid of patients suffering from meningitis, syphilis, and general paralysis of the insane precipitated gold sol, while normal fluid had no such effect. From this work he developed his famous test. The three zone curves which are now so well known were found by workin with progressive dilutions of cerebro-spinal fluid. The test is extremely valuable in differentiating the various forms of syphilis of the nervous system. The Lange test is of practically no value in diagnosis if used alone; it must be accompanied by the other findings on examination of the cerebro-spinal fluid and the physical and clinical examination of the patient before the true significance of the Lange test becomes apparent. Tests for globulin, total proteins, cell count and type, chlorides, and a serologic test, as well as the gold sol test (Lange) are run on cerebro-spianl fluid. (2).

A negative reaction may be as helpful in diagnosis as a positive reaction. The amount of fluid available will limit the number of tests performed, but, since it only requires 0.2 cc. of fluid the Lange test can usually be included. (12).

In the Hinton test for syphilis, according to Davies (3), the addition of various colloidal substances, such as egg albumin, human serum, 5% gum ghatti, 30% gum acacia, or 10% gelatin in proper proportions to spinal fluid markedly increased the sensitivity of the test. Equal parts of 20% gum acacia (or 5% gum ghatti) and Hinton negative human serum gave even better results.

The specific agents concerned in the precipitation of a sol-

ution of colloidal gold by certain pathologic spinal fluids remain, twenty-five years after Lange's discovery, a matter of controversy. The assumption that the mechanism is one of neutralisation of negative charges carried by finely dispersed colloidal particles with a subsequent precipitation of the neutral aggregate is agreed to by most workers. When one of the colloid components of the system is in excess, a protective action is observed both against spontaneous and electrolytic flocculation. This phenomenon was exhaustively studied by Zsigmondy. He measured the protective action of certain colloids, notably proteins, on the precipitation of gold sols, and found the degree of protection to be specific for each protein studied.

Most workers regard proteins in normal spinal fluid as the agents responsible for the precipitation of colloidal gold. Weston, Mellanby, and Anwyl-Davies believed the essential factor to be a globulin, since precipitation should not occur after removal of the globulin by $(NH_4)_2SO_4$. Cruickshank was able to simulate pathologic gold curves with suitable mixtures of albumin and globulin. It seems therefore, that proteins are the agents, alone or with inorganic radicals, which cause characteristic gold precipitation curves in abnormal spinal fluids. This work, while not furnishing absolute confirmation of this hypothesis, has yielded some highly suggestive results when considered in conjunction with the findings of other workers. (13).

The colloidal gold test is valuable but certain difficulties make it questionable. Preparation intricate, stability varies, reading calls for keen color sense and it is difficult to be sure that dif-

ferent lots have the same sensitivity. Colloidal carbon is new (colloidal mastic and colloidal benzoin are also used) and has advantages over others in ease of preparation, keeping qualities and in ease of reading the reaction. (4).

THEORY AND METHODS

1. Preparation of gold sol for use in the Lange colloidal gold reaction, price Method. (10). The technique is simple and had the advantage of definite standardization of sensitivity.

Preparation: To 500 cc. distilled water in a one liter flask add 5 cc. of 1% neutral $K_2C_2O_4$ (Potassium oxalate) and 2.5 cc. N/100 NaOH. Boil for two minutes. Add 2.5 cc. 1% gold sodium chloride ($AuCl_3NaCl.2H_2O$) drop by drop while boiling from a 1 cc. pipette. (Time 2 minutes).

Standardization: Have two racks of seven tubes each, one behind the other; to each tube in the back row add 6 cc. gold sol as prepared above. Working from left to right and omitting the first tube add varying amounts of N/100 HCl, beginning with 0.05 cc., 0.1, 0.15, 0.2, 0.25, and 0.3 to each tube. Mix tubes and stand 2-3 minutes. To each tube in the front row add 1 cc. of a normal cerebrospinal fluid diluted 1:160. Working left to right transfer 5 cc. gold sol in each tube in back row to each tube in front row. Mix and stand for 10 minutes. The end point is the greatest amount of acid added to 6 cc. of gold sol, 5 cc. of which on addition to 1 cc. of diluted C.S.F. (1:160) shows no color change. Usual readings, 000 (pink), 3 (lilac), 444(blue) and indicates that 0.1 cc. n/100 HCl should be added to each 6 cc. of gold sol used in the test.

2. Preparation of gold sols for the Lange test, method of Pennycuick, Woodcock and Cowan. (9).

Preparation of the formaldehyde-reduced sol: Heat 500 cc. of once or twice distilled water in a one liter beaker over a hot burner. When the temperature is 50-60°C., add 5 cc. of 1% auric chloride

and 5 cc. of 2% K_2CO_3 solution and stir the mixture. When temperature has almost reached boiling point, add 2.5 cc. of stock phosphorus reduced sol and stir. At boiling add 5 cc. 1% formalin (or 5 cc. 0.4% formaldehyde) and stir continuously. (Add formaldehyde, 5 drops, wait 1 minute, 5 drops and wait until color develops, 5 drops, wait, 10 drops, becomes lighter and add rest at once). The pH should be 6.5-7.25. The pH rises but never falls on standing.

Preparation of stock phosphorus reduced sol used for seeding: Prepare fresh every 6-8 months. Prepare a saturated solution of phosphorus in pure ether and dilute with 4 volumes of ether; stopper, keeps for years. To 500 cc. of distilled water at room temperature add 10 cc. of 1% auric chloride solution and 7.5 cc. of 2% K_2CO_3, mix, and stir 5 cc. ethereal solution of phosphorus into mixture. The solution turns brown. After 24 hours or 15 minutes boil and turns deep, clear, cherry red.

3. Preparation of gold sols, method of Glasoe and Sorum, (5).

Reagents: Purifiy the water used in preparation of gold sols by distilling it twice in a Pyrex still from a dilute solution of $KMnO_4$. (Use this to make up all solutions). 1. 1% chloraurie acid prepare by dissolving one 15 grain ampoule of Mallinckrodt's $HAuCl_4.3H_2O$ in 100 cc. water. 2. 2% K_2CO_3 prepared by dissolving 10 grams of Mallinckrodt's anhydrous K_2CO_3 in water to make 500 cc. of solution. 3. 1% formaldehyde prepared by dissolving 13.5 cc. of 35% formalin in water to make 500 cc. of the solution. 4. 0.01 M resorcinol prepared by dissolving 1.1 gm. resorcinol in 1000 cc. of water. 5. 0.2%

Chlorauric acid prepared by diluting 20 cc. of 1% with 80 cc. water.

6. 0.4% K_2CO_3 prepared by diluting 20 cc. of 2% with 80 cc. water.

7. 0.2% formaldehyde prepared by diluting 20 cc. of 1% with 80 cc. of water.

"Primary" nuclear sol prepared as follows: Mix 100 cc. of cold distilled water with 5.0 cc. of 0.4% K_2CO_3 and 5.0 cc. of 0.2% chlorauric acid, slowly and with constant stirring. The reduction takes place immediately, an amber colored sol being formed. When preserved in well stoppered Pyrex containers, this primary nuclear sol is unchanged over a period of six months.

"Sedondary" nuclear sol prepared as follows: Heat a mixture of 5 cc. of 0.4% K_2CO_3 and 5.0 cc. of 0.2% chlorauric acid just to boiling. Then add to 100 cc. cold distilled water 10 cc. of "primary" sol and stir the mixture. Stir two cc. of 0.2% formaldehyde into this mixture and allow the solution to stand at room temperature until the sol forms. When preserved in well stoppered Pyrex containers the primary sol is unchanged over 6 months.

"Final" sol for use in the tes is prepared as follows: Heat mixture of 3.5 cc. of a 2% K_2CO_3 and 5.0 cc. of 1% chlorauric acid just to boiling and add 480 cc. of cold water. Stir into this mixture 5 cc. (volume may be varied) of the secondary sol, followed by 2.5 cc. of 1% formaldehyde. On standing at room temperature an orange-red sol forms slowly.

Discussion: Variation in particle size with volume of secondary clear sol.

cc. of secondary sol	Average particle size in millimicrons (counted with ultramicroscope)
1.25	43
2.5	41
5.0	36
10.0	30
15.0	23.5

As the volume of "secondary" nuclear sol increases the average particle size decreases. Sols of large partical size were a murky amber yellow, intermediate size - clear orange yellow, sols of small particle size - brilliant wine red.

The average particle size of gold sol has a great influence on test readings. For a given pH, as the average particle size of the gold sol increases, the Lange test shows a greater degree of precipitation. As the average particle size increases, the sensitivity of the sol in the Lange test increases and vice versa.

The hydrogen ion concentration of gold sol also influences the test readings. pH of gold sol can be varied, by varyin amount of K_2CO_3 or formaldehyde used in preparation, by exposing to air, by adding dilute HCl or KCl to sol. Results show that as pH of the sol decreases, its sensitivity in the Lange test increases. Both pH and particle size must be carefully controlled to use in Lange test.

Increase in particle size gives more sensitive sol. Increase in pH gives a more stable sol. However pH should not go below 5 and particle size should not go above 40 millimicrons or readings tend to become erratic. Ideal (undecided as yet) about - small size and low pH (about 6).

Explanation of why the sensitivity of a gold sol is dependent

upon particle size and pH would appear fairly simple. It is well known fact that, other things equal, large particles are less stable than smaller particles. Hence, an increase in particle size should give a more sensitive sol. Gold sols are negatively charged, due, doubtless, to adsorption of negative ions. Hence, an increase in the concentration of negative hydroxyl ions (increase in pH) should increase the stability of the sol. All diseases in the first are affected in same manner as paresis.

4. Preparation of colloidal gold solution by Klass (8).

Reagents: 1. 1% solution of potassium oxalate, prepared just before use, 2. 1% solution of gold chloricde, 3. stock solution of 0.1 N NaOH, 4. stock solution of 0.1 N HCl, 5. Edestin solution (H.P., Pfanstiehl), 1:400 in 10% sodium chloride. Prepare the solution by transferring 0.25 grams edestin to a 100 ml. volumetric flask and making to volume with 10% sodium chloride. Shake the mixture at frequent intervals for a period of two hours and then filter. Preserve this solution in the refrigerator, and just before use, measure 1 cc. into a 25 cc. volumetric flask and dilute to the mark with distilled water, 6. 1% solution of alizarin red indicator in 50% alcohol, 7. Triple distilled water for all subsequent operations.

Method: Make a preliminary titration as follows each time that a new solution of gold chloride is prepared: To 50 ml. water add 0.5 cc. potassium oxalate solution and 0.5 ml. gold chloride with thorough mixing. Then introduce 5 cc. of this solution into each of Pyrex test tubes. To the first add 0.05 cc. of 0.02 N NaOH, to the second, 0.10 cc., to the third, 0.15 cc., and so on through the

eight. Put the whole series immediately into a 250 cc. beaker containing 75 cc. water. Bring the water to boiling in 2 minutes and boil 1 minute. Remove the tubes at once and examine. Usually there is just one solution that exhibits the characteristic orange color. Use the volume of alkali added to it, multiplied by 100 for the bulk preparation. The amount required is usually 20 to 25 cc.

To 560 cc. of water contained in a one-liter Erlenmeyer flask add with shaking 5 cc. of potassium oxalate solution, 5 cc. of gold chloride, and the volume of 0.02 N NaOH necessary in the preliminary titration. Cover the flask with a small beaker to protect from dust, and bring the mixture to boiling as quickly as possible, or 5 to 7 minutes. After 5-10 seconds of boiling, remove the flask from the flame. If all conditions are perfect, the rebound from the deep purple color to the final orange-red comes at the boiling point. Sometimes, however, the rebound does not come for several minutes, or perhaps for several hours. After the solution has cooled to room temperature, a 5 cc. aliquot is removed. To this add 2 drops of the alizarin red indicator, and then 0.001 N HCl from a graduated pipette until the neutral point is reached, as indicated by a change to a brownish red color. On the basis of this titration, calculate the volume of 0.02 N HCl necessary for the neutralization of the stock solution. Add this slowly with constant shaking.

Now test the sensitivity of the gold sol with the freshly diluted edestin solution. Add 5 cc. of colloidal gold to each of a series of 6 test tubes containing 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 cc. of the edestin. If the gold is found to have the correct sen-

sitivity, the readings after about 18 hours will be 0, 1-2, 3, 4.5, 5, 5. A final check can be made with paretic and negative cerebrospinal fluids. The edestin serves as a primary standard which permits the adjustmen of successive preparations to very neady the same sensitivity.

5. Preparation of colloidal gold solution, method of Mellanby an Anwyl-Davies. (1).

Reagents: 1. Merck's blue label gold chloride, 1gm. in 100 cc. water. 2. Neutral potassium oxalate, 1 gm in 100 cc. water, 2. Potassium Hydroxide, 1 gm. in 100 cc. water, 4. Doubly distilled water. Adjust the gold chloride sdution by neutralising the excess acid. Set up 6 tubes, each containing 1 cc. of gold chloride solution. Add to each tube, in order, the following amounts of 1% potassium hydroxide solution: 0.6 cc., 0.5 cc., 0.4 cc., 0.2 cc., and 0.1 cc. To each tube add 2 drops 1% phenolphthalein in 95% alcohol. The tube having the optimum reaction will be the most turbid (usually the tube with 0.3 cc. alkali). Add to each tube 1 cc. of 1% neutral potassium oxalate solution. Note the largest amount of alkali that can be added and still have complete reduction (dense black precipitate) in 5 minutes. This is usually tube 4 containing 0.3 cc. alkali. Add the proper amount of potassium hydroxide solution indicated by the titration to the gold chloride solution.

To 100 cc. of doubly distilled water, add 1 cc. neutral potassium oxalate solution, heat to boiling and add, drop by drop, gold chloride solution, 1 cc. plus the amount of alkali added (e.g. a total of 1.3 cc., if 0.3 cc. of alkali has been added). A deep orange red (not bluish) solution should develop.

6. Colloidal gold test, according to Bray. (1).

Method: Cleanse glassware with white (Ivory, etc.) soap and water, then cleansing fluid; then rinse with running water and finally with distilled and doubly distilled water. Sterilise in hot air. Place ten test tubes in a rack. Into the first place 1.8 cc. of 0.4% sodium chloride solution and into each of the others 1 cc. Centrifuge and add 0.2 cc. clear spinal fluid to the first tube and mix. Transfer 1 cc. to the second tube and mix. Continue in this manner, discarding 1 cc. from the tenth tube. Add to each tube 5 cc. colloidal gold solution and mix. Let stand overnight at room temperature. Each time, carry the following controls in one rack as tubes 11 and 12. In tube 11 put 1.7 cc. of 1% sodium chloride solution and in tube 12 put 0.5 cc. Add 5 cc. colloidal gold solution to each.

Record results on basis of 1 to 5:

0. No change
1. Bluish red
2. Reddish blue
3. Blue
4. Pale blue
5. Colorless

Significance: Normally there is no change over 1 in any tube; for example, 0001100000. Marked change in the first three tubes (Zone I reaction) occurs in general paresis (with a positive spinal fluid Wassermann) and in active disseminated sclerosis (with negative Wassermann); for example, 5554321000. The greatest change in the fourth and fifth tubes (Zone II reaction) occurs in tabes and in meningovascular syphilis, and sometimes in multiple sclerosis, encephalitis lethargica, and poliomyelitis; for example, 1124320000, the change rarely being above 3 or 4. The greatest change in the last three

tubes (Zone III reaction) occurs in pyogenic meningitis; for example, 0012234553. Many nonsyphilitic fluids with increased protein (old hemorrhage, tumors, etc.) give positive reactions. In tuberculous meningitis the greatest change is usually between Zones II and III; for example, 0000243000.

CONCLUSIONS

Colloidal gold today is prepared by three methods: the Eike method, using glucose, the Miller method using oxalic acid and formaldehyde, and the Mellanby method using potassium oxalate. The glucose method (Eike) yields beautiful preparations but they are not especially sensitive. The solutions produced by the Miller method are very sensitive. The Mellanby method is not suitable for preparations of large quantities.

Satisfactory preparations are rose red, cherry or salmon red by transmitted light with no trace of blue tint. By reflected light the color is clear brown. The pH should be between 6 and 7. The difficulties in preparing gold sols for use in the Lange test may be ascribed to lack of reproducibility of the reagents. Potassium carbonate and gold chloride contain variable amounts of absorbed water. The formaldehyde and glucose solutions often vary in strength and acidity. This difficulty may be eliminated by using a neutral solution of potassium oxalate which may be standardized to account for the variations in the gold chloride. A standard alkali should be used to regulate pH and a standard acid for final adjustment to the proper or required sensitivity.

The Lange test is a definite aid in diagnosis and is a valuable means of following the progress of diseases of the central nervous system. The use of the photelometer in making Lange test readings gives more accurate results. However for practical clinical work there is no advantage since the general forms of the curves and the relative colors of the solutions are more important than the actual colors.

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